Identification of potential new client proteins of Cdc37 in fission yeast

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Ph.D Thesis
University of Edinburgh
December 2006

Abstract

Cdc37 is a molecular chaperone whose clients are predominantly protein kinases. Chaperones form an essential component of the cell cycle. Many kinases rely on chaperones for folding and/or activation. Chaperones maintain their clients in an activation competent state which prevents their degradation. Synthetic lethal genetic screen was carried out to identify and characterize novel client proteins of Cdc37 in *S. pombe.*

During the screen, 10 strains were picked out of 40,000 colonies as potentially synthetically lethal with *cdc37-681*, a temperature-sensitive allele, at permissive temperature. A genomic library was transformed into each synthetic lethal mutant. Plasmids that rescued the mutants were recovered and sequenced. *wis4*, *msc1*, *nak1* and *cdc7* were identified as candidate genes that rescue the various mutants. *wis4* encodes a MAP kinase kinase kinase involved in the stress-responsive signal transduction pathway. *msc1* is a multi-copy suppressor of *chk1* and has a role in regulating chromatin structure. *nak1* encodes an essential protein kinase and plays a role in the regulation of cell polarity, growth and division. Cdc7 is a protein kinase essential for septation and cell division.

A known c*dc7ts* mutant, *cdc7-24*, was shown to be synthetically lethal with *cdc37-681*. The synthetic lethal mutation outcrossed from J322 (the strain rescued by $cdc7^{+}$) shows the same phenotype as *cdc7-24*. Further genetic analysis indicates that the mutation of J322 is in *cdc7*. Therefore Cdc7 is a possible Cdc37 client, but Cdc7 protein levels do not change in *cdc37-681* or *cdc37-184* at restrictive temperature. Cdc7 kinase activity is reduced in *cdc37-681* and *cdc37-184*, indicating that Cdc37 function is needed for Cdc7 kinase activity. The cell morphology of *cdc37ts* in combination with GFP-tagged Cdc7 is similar to that of *cdc7ts* mutants and differs from the *cdc37ts* single mutant. It suggests the interaction may happen between compromised Cdc7 and Cdc37. Cdc7 can locate to the spindle pole body properly in *cdc37ts* strains, which suggests Cdc7 localization does not require Cdc37.

Acknowledgements

I would like to thank to my supervisor Peter Fantes, who gave me the opportunity to do my PhD in his lab under his expert guidance. Thanks the Darwin trust of Edinburgh for funding, I have learnt a great deal and have had a wonderful time in Edinburgh. Thanks to Ina Martin and Emma Turnbull for their help when I joined the lab. Thanks to Dr Kevin Hardwick and Stuart Macneil for use of their equipment and help for protein work. Thank you to Josefin Fernius for advice on kinase assays and Karen May for suggestion on microscopy technology. Thanks for the plasmids and strains from Dr Klaus Leonhard in Professor Paul Nurse's lab, Dr Viesturs Simanis and Dr Nancy Walworth.

Thank you to my family, Dad and Mum for being so supportive and encouragement throughout.

Abbreviations

BSA	Bovine serum albumin
Cl	chloride ion
℃	degrees Celsius
DAPI	4,6,diamidino-2-triphosphate
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
EDTA	ethylenediaminetetraacetic acid
g	gram
K	potassium ion
l	liter
LB	Luria-Bertani medium
M	molar
Mg	magnesium ion
mg	milligram
ml	milliliter
mM	millimolar
μg	microgram
μl	microliter
μM	micromolar
Na	sodium ion
OD	optical density
PCR	polymerase chain reaction
SDS	sodium dodecyl sulphate
Tris	tris aminomethane
Ts	temperature-sensitive

Ura	uracil
Leu	leucine
MOPS	4-morholinepropane sulphonic acid
EMM	Edinburgh minimal media

1 Introduction

1.0 Molecular chaperones

1.1 Introduction

Proteins are central to all biological processes. Protein is translated from RNA template by ribosomal complexes, producing a linear nascent polypeptide chain. To become functionally active, newly synthesized polypeptide chains must fold into unique three-dimensional conformations, based on the information encoded in their amino acid sequences (Dobson, 2004). A group of proteins, known as molecular chaperones, are essential for protein folding to occur with high efficiency in both prokaryotic and eukaryotic systems. A molecular chaperone acts as the protein that helps in the folding of a second protein, typically through cycles of binding and release, but does not form part of the final native structure (Young et al., 2004). The proteins on which molecular chaperones act are referred to as "client proteins". The main role of molecular chaperones is to prevent protein misfolding and aggregation, and maintain client proteins in a competent state. In some cases, molecular chaperones even recognize incorrectly folded proteins and present them for degradation (Horwich et al., 1999).

Table 1.1 Molecular chaperones in different organisms

Group	**Family**		**Mammalian**	**Budding yeast**	**Fission yeast**	***E. coli***
Folding chaperones	Hsp100					ClpA
			Hsp100	Hsp104	N/A	ClpB
						ClpC
	Hsp90		Hsp90	Hsp82	Hsp90	HtpG
			Grp94	Hsc82		
	Hsp70		Hsc70	Ssa1	N/A	DnaK
				Ssb1/2	N/A	DnaJ
				Bip/GRP78	N/A	GrpE
	Hsp60		Chaperonin	Cpn60	N/A	GroEL
	Hsp40		N/A	Sis1	N/A	N/A
			Hdj1	Ydj1	N/A	N/A
co-chaperones	Cdc37		p50	Cdc37	Cdc37	N/A
	p23		p23	Sba1	N/A	N/A
Scaffold chaperones	Hop		Hop	Sti1	Sti1	N/A
Other chaperones			N/A	N/A	N/A	Trigger Factor
			Bag-1	N/A	N/A	N/A

1.2 Brief introduction of the main molecular chaperone systems

1.2.1 The Hsp70 system

Hsp70s are found in both prokaryotic and eukaryotic systems. Its members represent the most highly conserved molecular chaperones. Hsp70 acts on nascent chains and is involved in the prevention of protein aggregation as well as protein targeting and translocation, and protein degradation (Flaherty et al., 1991; Pilon and Schekman, 1999). Hsp40 proteins interact with Hsp70 by modulating its ATPase activity to achieve these diverse functions(Cyr et al., 1994).

Hsp70 chaperones have two major functional domains, an N-terminal ATPase domain and a C-terminal peptide-binding domain (Flaherty et al., 1991; Zhu et al., 1996). Hsp70 functions are carried out through an ATP-regulated cycle of substrate binding and release. In bacteria, the cycling of Hsp70 homolog DnaK between the ATP- and ADP-bound states is regulated by DnaJ and GrpE (Szabo et al., 1994). DnaJ positively regulates the ATPase activity of DnaK and recognizes hydrophobic peptides and presents unfolded polypeptides to DnaK. GrpE induces the dissociation of ADP from DnaK, leading to substrate release (Sreeramulu et al., 2005).

The Bag-1 has been identified as a nucleotide-exchange factor and specific regulator of Hsp70 in the eukaryotic cytosol (Hohfeld and Jentsch, 1997). It contains

an additional ubiquitin-like domain and cooperates functionally with CHIP (Takayama and Reed, 2001), which is a TPR clamp (Abbas-Terki et al.) co-chaperone of Hsp70 and Hsp90. CHIP promotes ubiquitination and proteasomal degradation of Hsp70- and Hsp90-bound substrates in vivo (Luders et al., 2000).

1.2.2 The Hsp90 system

Hsp90 chaperones act downstream of the Hsp70/Hsp40-chaperone system and play an important role in maintaining client proteins in competent state. Hsp90 interacts with different kinds of substrates, such as steroid hormone receptors, protein kinases and cell-cycle regulators (Abbas-Terki et al., 2002). Substrate recruitment occurs in an Hsp70-dependent manner (Hutchison et al., 1994) and is mediated by Hop, which interacts directly with both Hsp70 and Hsp90 (Scheufler et al., 2000). Hsp90 undergoes regulated cycles of ATP binding and hydrolysis in the process of folding client proteins (Prodromou et al., 2000). Its function also relies on cooperating with several co-chaperones, depending on the substrate protein bound. Many of these co-chaperones possess TPR-clamp domains and compete for binding to the C-terminal EEVD peptide motif of Hsp90 (Prodromou et al., 2000), such as immunophilins Cyp40, FKBP51, FKBP52 (Pratt, 1998).

1.3 Client proteins and chaperones

The correct folding of the protein is essential for achieving its biological function (Dobson, 2004). The amino acid sequence of the nascent polypeptide contains

information to reach its native three-dimensional structure (Dobson, 2004). However protein folding as a spontaneous process is slow and often produces misfolded structures. To complete accurate efficient folding, molecular chaperones play a important role.

Different molecular chaperones usually act on specific groups of client proteins. They display high level of substrate specificity, recognising the client proteins based on their specific amino acid sequence. For example, *Ydj1* and *Sis1* are both Hsp40 chaperones in budding yeast. They have very different preferences in recognizing the client proteins (Westwood et al., 2004). *Ydj1* associates with polypeptides with a hydrophobic core of 3-4 amino acids adjacent to one anther, *Sis1* favours clients containing multiple hydrophobic residues which are aromatic or lysine (Westwood et al., 2004). Molecular chaperones also display substrate specificity based on the conformational state of the client protein. In *S.cerevisiae*, Ydj1 preferentially associates with non-native, unfolded polypeptides (Cyr, 1995). In contrast, DnaK in *E.coli* binds both short hydrophobic peptides in an extended conformation and some proteins in a native conformation (Wawrzynow and Zylicz, 1995). The ability of molecular chaperones to recognise specific client conformations creates a network of precise chaperone-client interactions.

Nascent polypeptide chains can form insoluble aggregate complexes, because of their hydrophobic nature (Barral et al., 2004). Molecular chaperones can bind nascent polypeptides, associating with them when they are translated from ribosomes. For example, the molecular chaperone Trigger Factor in *E.coli* scans the nascent polypeptide when it is being translated by the ribosome, and identifies and prevents hydrophobic patches of sequence from aggregating (Young et al., 2004). The Trigger Factor disassociates from the client polypeptide once the ribosome has left the

complexes (Hesterkamp and Bukau, 1996). Once client polypeptides have been translated and left the ribosome, another set of chaperones protect them from forming insoluble aggregates. In *S. cerevisiae*, *Ydj1* acts on the polypeptides and prevents them from forming insoluble aggregates (Cyr, 1995). If nascent polypeptides form aggregates for some reason, chaperones such ClpA, a Hsp100 family chaperone, can resolubilise inactivated and denatured clients (Parsell et al., 1994).

After client polypeptides have been translated and left the ribosome, they must be maintained by molecular chaperones in an activation competent state, ready for folding. For example, chaperone SecB maintains proOmpA in *E. coli* in a competent state (Lecker et al., 1990). Under heat stress, in *Xenopus* oocytes, small heat shock proteins bind denatured clients and hold them in a folding competent state until other chaperones fold them into their native state (Heikkila, 2004).

Folding of client proteins is carried out by ATP-dependent molecular chaperones. The cytoplasmic chaperonin requires ATP for activity. The folding reaction proceeds through ATP-independent formation of a binary complex, followed by ATP-dependent release of the native protein (Gao et al., 1992). In bacteria, the cycling of Hsp70 homolog DnaK between the ATP- and ADP-bound states is regulated by DnaJ and GrpE (Szabo et al., 1994). DnaJ positively regulates the ATPase activity of DnaK and recognizes hydrophobic peptides and presents unfolded polypeptides to DnaK. GrpE induces the dissociation of ADP from DnaK, leading to substrate release (Sreeramulu et al., 2005).

1.4 Chaperone Hsp90

Hsp90 was first identified as a 90 kDa protein whose RNA levels were dramatically increased upon heat shock (Finkelstein et al., 1982). Soon afterwards, Hsp90 homologues in other organisms were also identified. In mammalian cells, there are two isoforms for this protein, identified as Hsp90- α and Hsp90- β (Nemoto and Sato, 1998). Its homologues include Hsp86 and Hsp84 in mice (Hoffmann and Hovemann, 1988), Hsp83 in *Drosophila* (Konstantopoulou and Scouras, 1998), Hsc82 and Hsp82 in budding yeast (Kimura et al., 1994), and Swo1 in *S. pombe* (Aligue et al., 1994). Grp94 (94-kDa glucose-regulated protein), was identified as the additional version of Hsp90 in the endoplasmic reticulum of human cells, and is thought to exist in all eukaryotes (Gupta, 1995).

Hsp90 is one of the most abundant proteins in eukaryotic cells. It accounts for 1–2% of total protein in unstressed mammalian cells. When cells are heated, Hsp90 increases to 4–6% of cellular proteins (Goetz et al., 2003). Hsp90 gene sequence is known to be highly conserved among the species. Hsp90 sequence identity between human and budding yeast is 60% (Gupta, 1995). The Hsp90 isoforms of human cells, referred to as Hsp90- α and Hsp90- β , exhibit 99% identity in protein sequence (Gupta, 1995). It is evolutionarily conserved among species, and is proved essential for the cell survival.

1.4.1 Hsp90 domains

Hsp90s consist of two highly conserved domains: a 25kDa N-terminal domain and a 55 kDa C-terminal region, which are connected by a charged linker region. This linker region has a variable length and in composition in different eukaryotic species (Roe et al., 1999).

The N-terminal domain of Hsp90 contains an ATP binding site. The binding of ATP at the N-terminal site changes the conformational state of Hsp90 and is essential for its interactions with client proteins and co-chaperones (Siligardi et al., 2002). An Hsp90-specific drug, geldanamycin, can compete with ATP/ADP in the nucleotide binding pocket, disrupt Hsp90 function and result in degradation of Hsp90 client proteins by ubiquitin-dependent proteasome pathway (Schnaider et al., 1998).

The peptide sequence MEEVD at the end of C-terminus of Hsp90 is highly conserved among all eukaryote cells. This peptide is critical for the binding to various TPR Hsp90 co-chaperones (Pratt and Toft, 2003). The tetratricopeptide repeat present in Hsp90 co-chaperones binds to the C-terminal MEEVD motif, and these interactions help in forming the chaperone complex.

1.4.2 Hsp90 client proteins

Hsp90 associates with a wide range of client proteins which are involved in various cellular processes including signal transduction, protein folding and degradation. Hsp90-associated proteins can be categorized into three general groups: protein kinases, transcription factors/polymerases and co-chaperones.

1.4.2.1 Protein kinases

The Src (Rous sarcoma virus, $p60^{src}$) family of tyrosine kinases is involved in signal transduction following growth factor stimulation and integrin-mediated cell-substrate adhesion in mammalian cell (Jin et al., 1999). It has been used as a model client of molecular chaperones in mammalian cells and when artificially expressed in yeast. In budding yeast, mutation in Hsp90 gene will result in reduction of both protein level and kinase activity of v-src. It has been suggested that Hsp90 stabilizes the v-src and affects both its activity and specificity (Lamphere et al., 1997).

In *S. pombe*, Wee1 protein kinase negatively regulates entry into mitosis by mediating the inhibitory tyrosine phosphorylation of Cdc2-cyclin B kinase (Den Haese et al., 1995). Hsp90 is required for the assembly and/or disassembly of the functional Wee1 protein complex (Aligue et al., 1994). Impairing Hsp90 function by treatment with geldanamycin causes reduction in cellular level of the Wee1 kinase activity and the protein becomes rapidly degraded by the proteasome, indicating that Wee1 requires Hsp90 for its stability and maintenance of function (Goes and Martin, 2001).

Raf-1 is part of a conserved signal transduction pathway that transmits signals from cytosolic and transmembrane tyrosine kinases to mitogen-activated protein kinases (Galaktionov et al., 1995). In *Drosophila*, Raf-1 kinase associates with the Hsp90 chaperone complex containing $p50^{Cdc37}$. Formation of Raf-1-Cdc37-Hsp90 complex is crucial for Raf-1 activity and MAPK pathway signalling (Grammatikakis et al., 1999a). Activation of Raf-1 by expression of Ras in insect cells is strongly inhibited by dominant negative Cdc37 or by geldanamycin. It indicates that Hsp90 is essential for Raf-1 stabilization (Grammatikakis et al., 1999a).

1.4.2.2 Transcription factors

Steroid hormone receptors are ligand-dependent transcription factors. Steroid hormone receptors are known to be complexed with Hsp90. Steroid hormone receptors are associated with the Hsp90 chaperone complex in the cytoplasm prior to ligand binding. This association allows steroid hormone receptors to achieve a structural conformation that is competent for ligand binding (Neckers, 2002). Treatment with geldanamycin will disrupt Hsp90 complexes and prevents the assembly of the complexes required to maintain hormone receptors in their mature, ligand-binding configuration (Krone, 2003).

1.4.2.3 Hsp90 co-chaperones

Processing of the substrates by the Hsp90 molecular chaperone system involves complex interactions between Hsp90 and its co-chaperones. Many Hsp90 co-chaperones are characterized by the presence of tetratricopeptide repeat domains, which mediate binding to Hsp90 (Scheufler et al., 2000).

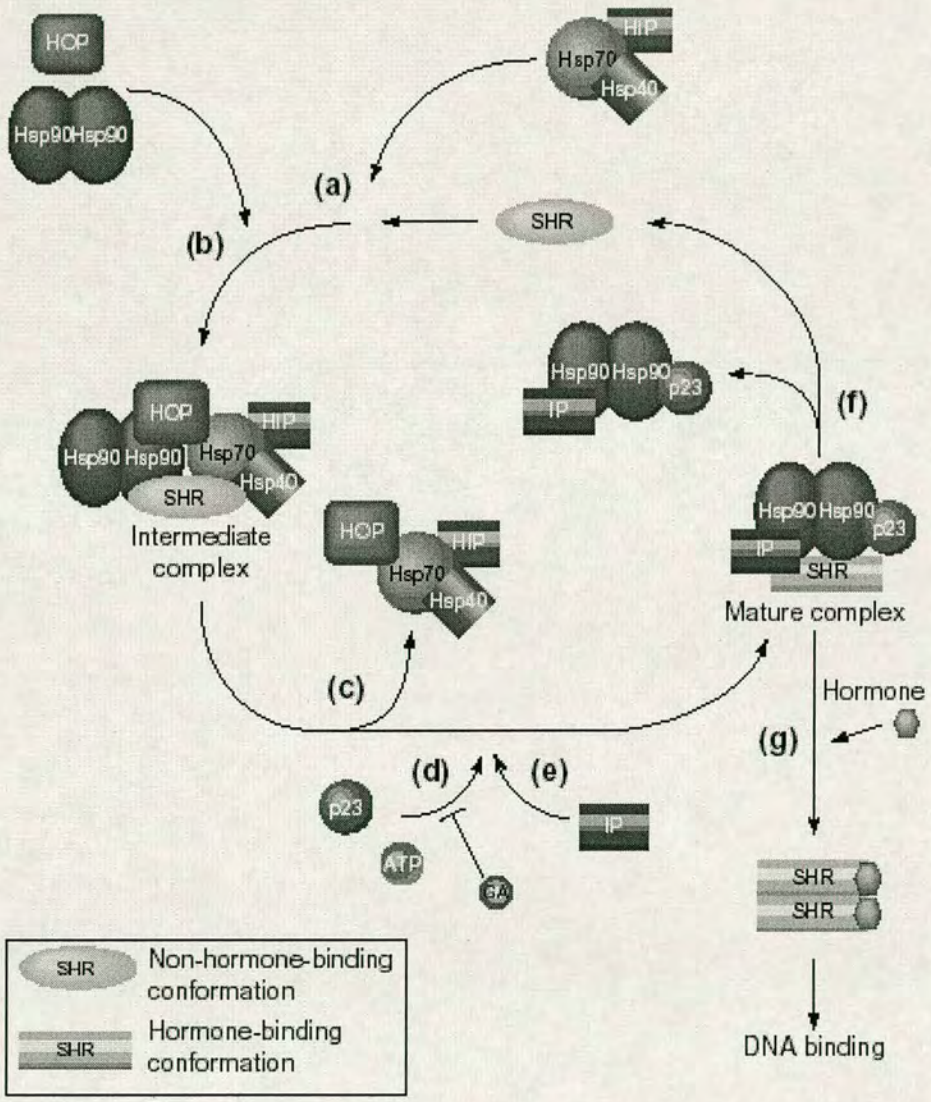
Hop (Sti1 in *S.cerevisiae*) can bridge Hsp90 and Hsp70, via two sets of TPR domains (Scheufler et al., 2000). TPR-containing immunophilins, FKBP52 and FKBP51, the cyclosporin A-binding immunophilin CyP40, and protein phosphatase PP5 have also been identified in Hsp90 complexes (Riggs et al., 2004). The TPR domains of these proteins have similar X-ray crystallographic structures and target co-chaperone binding to the MEEVD sequence of Hsp90 (Riggs et al., 2004). In *Saccharomyces cerevisiae*, Cns1, a Hsp90 co-chaperone, acts as a *bona fide* co-chaperone which interacts with TPR co-chaperones and is essential for viability (Tesic et al., 2003). TPR co-chaperones play an important role in the function of

Hsp90 *in vivo*. The maturation of the glucocorticoid receptor (GR) revealed the requirement for Cpr7 and Sti1 for full Hsp90 activity (Tesic et al., 2003).

1.4.3 Hsp90 chaperone activity in holding substrate

Steroid hormone receptors act as the ligand dependent transcription factors to mediate hormone signaling. Studies show that the members of the steroid hormone receptor family require chaperones to build conformation state which is capable of binding to ligand. Hsp90 was identified as one of important components which plays an important role in steroid hormone receptor's structural maturation and function (Pratt, 1992). Steroid hormone receptors pass through several chaperone complexes before being functional. The initial interactions involve Hsp70 and its co-chaperone Hsp40 and Hip. Hsp90 co-chaperone Hop acts as the scaffold protein which brings Hsp70 chaperone elements together with Hsp90 to form the intermediate complex with immature steroid hormone receptor (Hutchison et al., 1994). The Hsp70 components dissociate from the intermediate complex soon afterwards. At the same time, two Hsp90 co-chaperones, p23 and an immunophilin, enter the complex to help Hsp90 to fold the steroid hormone receptor into proper conformational state

Figure 1.1 Hsp90 chaperone cycle involved in steroid-hormone receptor activation (Buchner, 1999)**(a)** Initial interactions require only Hsp70 and cofactors. **(b)** The scaffold protein Hop brings together elements of the Hsp70 and Hsp90 to form the intermediate complex.**(c–e)** The Hsp70 components dissociate and, at the same time, p23 and one of the large immunophilins (IP) enter the complex. **(f)** The SHR is spontaneously released from this complex. **(g)** It can then bind hormone, dimerize and bind DNA.



(Johnson and Toft, 1994). The matured steroid hormone receptor is released from this complex when the hormone binds (Figure 1.1).

1.5 General introduction about Cdc37

Cdc37 was first identified as a temperature-sensitive cell division cycle mutation that arrests cells in G1 at START in *Saccharomyces cerevsiae* (Reed, 1980). Then the nucleotide sequence of Cdc37 in budding yeast was presented (Ferguson et al., 1986). Cdc37 homologues in other organisms were also identified. In *Drosophila* cells, the mutations in Cdc37 and Hsp83 affect the signaling transduction pathway by the sevenless receptor tyrosine kinase (Cutforth and Rubin, 1994). Biochemical analysis found that a 50kDa protein which is associated with v-src and Raf-1 kinase is homologous to Cdc37 in chicken cells (Ozaki et al., 1995). Diverse protein kinases were identified as clients of the Cdc37 protein. In *S. cerevisiae* Cdc37 plays a regulatory role in the association of Cdc28 with cyclins (Farrell and Morgan, 2000; Gerber et al., 1995). In mammalian cells, $p50^{Cdc37}$ is found to be a subunit of a Hsp90 complex that stabilizes Cdk4 and Cdk6 (Dai et al., 1996; Lamphere et al., 1997; Stepanova et al., 1996). Cdc37 is required for maintenance the kinase activity of v-src (Dey et al., 1996; Perdew et al., 1997) and Raf-1 (Grammatikakis et al., 1999c; Silverstein et al., 1998). It was proposed to act as a molecular chaperone, which is required for stability and/or activity of some kinases. Cdc37 was usually isolated in a Hsp90 complex and in association with Hsp90 clients (Basso et al., 2002; Siligardi et al., 2002) and it is thought to be a Hsp90 co-chaperone. Cdc37 serves a role to deliver client proteins to Hsp90 complex for folding (Prodromou and Pearl, 2003).

1.6 Cdc37 sequence

Cdc37 homologues have been found in many organisms from yeast, fungi, metazoa and human. An alignment of Cdc37 sequences shows very low similarity to one another in different organisms (Fig1. 2). The overall level of identity of the *S. pombe* to metazoan proteins is around 21–23% (Westwood et al., 2004), while Cdc37 gene's similarity between human and chick is 84% (Huang et al., 1998). The extreme N-terminal sequences of Cdc37 are highly conserved among species. The first 40 N-terminal amino acid sequence displays 80% identity between *S. pombe* and *S. cerevsiae* and 50% identity between human and yeasts (Westwood et al., 2004). The function of Cdc37 homologues may be conserved in different species rather than the sequence.

1.7 Cdc37 domains

Human Cdc37 can tentatively be divided into 3 domains: the N-terminal domain, the middle domain and C-terminal domain (Scroggins et al., 2003; Shao et al., 2003a). The N-terminal domain covers amino acids 1-126, the middle domain consists of amino acids 126-282, and the C-terminal region is composed of residues 283-378 (Shao et al., 2003a). The N-terminal domain is required for client binding. Cdc37 binds directly with client proteins such as Raf (Silverstein et al., 1998), v-src (Lee et al., 2002), MOK (Miyata, 2004), Lck (Prince and Matts, 2004) through the catalytic domain of the kinases. The first 8 amino acids at the N-terminus are essential for client binding (Shao et al., 2001). The Hsp90-binding domain of Cdc37 was narrowed down to a 120 amino acid mid-section (Shao et al., 2001) Based on

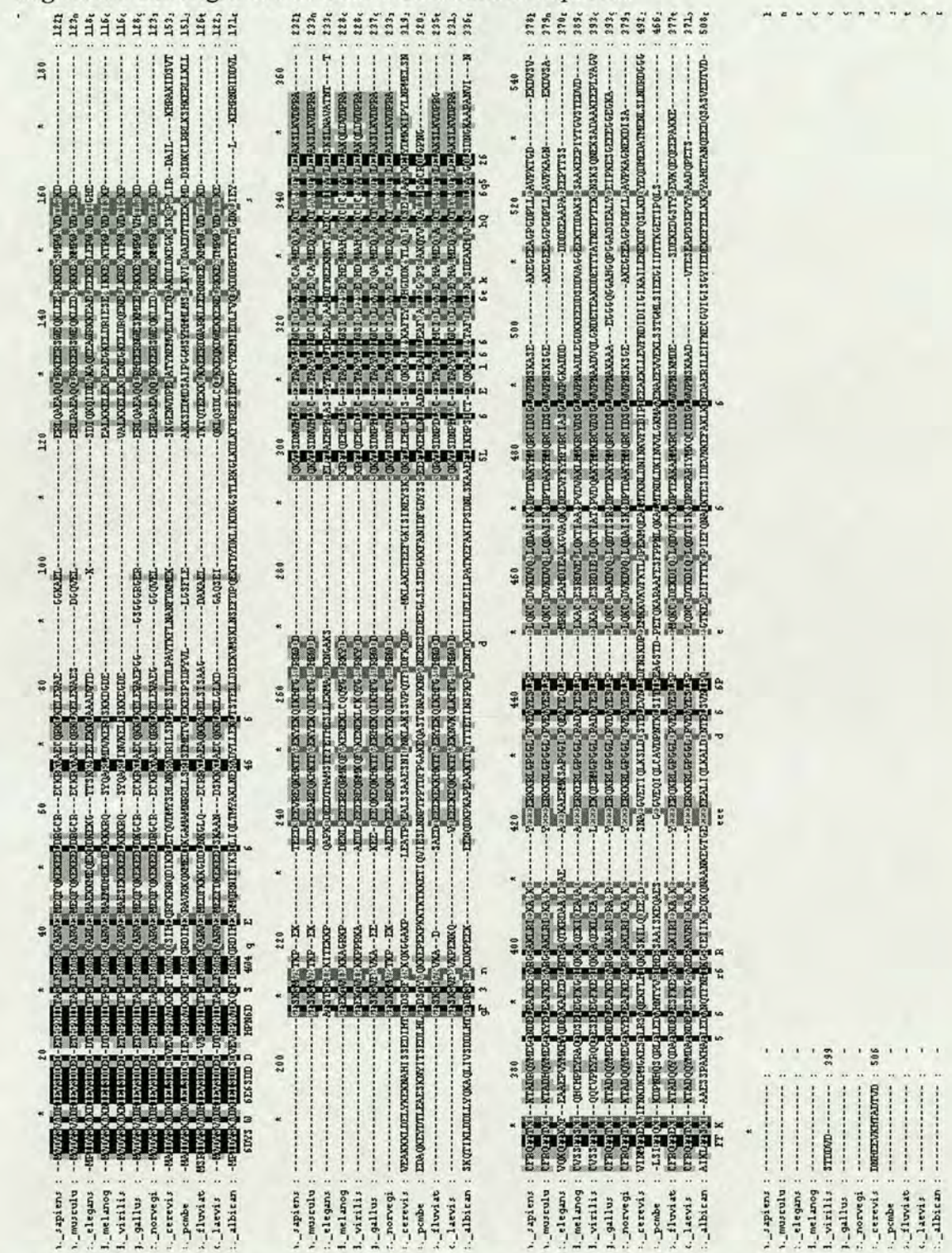
sequence similarity with human Cdc37, the putative Hsp90 binding site in budding yeast Cdc37 is proposed to lie between K243 and R343 (Shao et al., 2003a). Conversely, an N-terminally truncated mutant that retains the Hsp90-binding is still able to suppress the ATPase activity of Hsp90 (Piper et al., 2003). Remarkably, the C-terminal domain of Cdc37 in *S.cerevisiae* is dispensable for the function with respect to cell viability and v-src folding (Lee et al., 2002). The Cdc37ΔC-terminal mutant can restore v-src activity as effectively as full length Cdc37 protein (Lee et al., 2002). In *S. pombe*, deletion of the C-terminal 100 amino acids does not affect the growth of the mutant (Turnbull et al., 2005).

1.8 Crystallisation studies of Cdc37 structure

The structure of middle and C-terminal human Cdc37 was determined by crystallisation studies. The C-terminal fragment of $p50^{cdc37}$ consists of helical bundle structure. A large 6-helix at the N-terminal end (residues 148–245) and a disordered polypeptide chain (amino acids 309-315) was connected to a small 3-helix bundle (292–347) through a long helix (246–286) (Figrue1.3) (Roe et al., 2004).

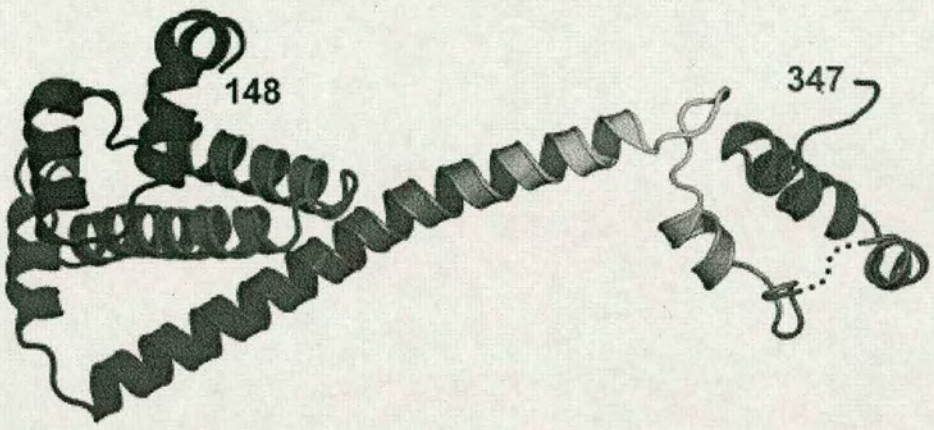
Crystallization studies also show that C-terminal truncated human Cdc37 forms a dimer. The center of the interaction involves close packing of the main chain and side chains of Gln 247 and Tyr 248, in the first turn of the long connecting helix, with their equivalents in the other monomer (Roe et al., 2004). This interaction of hydrophobic patches is strengthened by ion-pairs between Lys 240 and Asp 245 from separate monomers(Figrue1.4) (Roe et al., 2004).

Figure 1.2 An alignment of Cdc37 from different species.



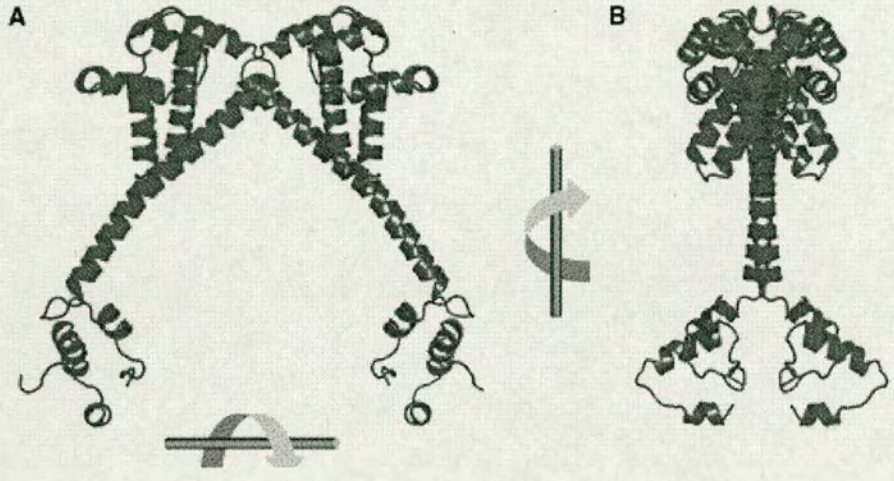
Black indicates codons identical between species and grey denotes amino acids of similar type

Figure 1.3 The crystal structure of human Cdc37 amino acid 148 to 347 (Roe et al., 2004).



A 6-helix bundle was identified, connected by a long single helix and a disordered polypeptide chain

Figure 1.4 Crystal structure of amino acid 148-347 of human Cdc37 forming a dimer (Roe et al., 2004)


A
B

1.9 Phosphorylation of Cdc37

A number of conserved serine residues lie within the extreme N-terminal domain of Cdc37, which exhibits a high level of homology among different organisms. Serine 14 and 17 in *S. cerevisiae* (Bandhakavi et al., 2003), serine 14 in *S. pombe* (Turnbull et al., 2005), serine 13 in rat (Miyata and Nishida, 2004a) and human (Shao et al., 2003b), have been identified as important sites for Cdc37 phosphorylation. The kinase Casein kinase II (CKII) can phosphorylate Cdc37 *in vivo* and *in vitro* (Miyata and Nishida, 2004b) (Shao et al., 2003a). Mutation of serine 13 residue to alanine in rat Cdc37 protein inhibits phosphorylation by CKII. At the same time, phosphorylation by CKII in serine 13 in rat, is required for Cdc37 activity. (Miyata and Nishida, 2004b). These observations show that Cdc37 and CKII maintain each other's activity through the feedback loop of activation (Bandhakavi et al., 2003).

Mutation of serine 13 residue to alanine reduces the binding affinity with some client proteins such as Akt, Aurora-B, Cdk4, and v-src *in vivo*. It also results in the reduction in protein levels of client protein MOK and Raf-1(Miyata and Nishida, 2004b). It suggests that phosphorylation of serine 13 of Cdc37 by CKII is important for client protein binding. Serine 13 is also essential for the recruitment of Hsp90 to protein kinase-Cdc37 complexes. Mutation of human Cdc37 serine 13 to alanine inhibits the interaction between Hsp90 and MOK-Cdc37 complexes or with Raf1-Cdc37 complexes, but the binding between Hsp90 and Cdc37 was not was not affected by the Ser13 mutations (Miyata and Nishida, 2004b).

1.10 Cdc 37: a Hsp90 co-chaperone

A variety of evidence demonstrates that Cdc37 acts as an Hsp90 cochaperone. Some protein clients are associated with both Cdc37 and Hsp90, for their folding, maturation, or stability. For example, LKB1 is a serine/threonine kinase tumour suppressor, and was found to interact with Hsp90 and Cdc37 to form heteromeric complex. Pharmacological inactivation of Hsp90 results in rapid degradation of LKB1 by the proteasome-dependent pathway. It suggests that the Hsp90/Cdc37 complex is a major regulator of the stability of LKB1 (Nony et al., 2003).

Cdc37 is the primary determinant of Raf-1 recruitment to Hsp90. Formation of a Raf-1-$p50^{cdc37}$-Hsp90 complex is crucial for Raf-1 activity and MAPK pathway signaling. Activation of Raf-1 by expression of Ras in insect cells was strongly inhibited by dominant negative $p50^{cdc37}$ or by geldanamycin. It indicates that Hsp90 is essential for Raf-1 stabilization. And Cdc37 is essential for Raf-1 recruitment to Hsp90 (Grammatikakis et al., 1999a).

In mouse fibroblasts, a primary target of Cdc37 is Cdk4. Cdk4 is activated by D-type cyclins and functions in passage through G1. Cdc37 is sufficient to recruit Hsp90 to Cdk4 both *in vitro* and *in vivo*. Cdc37/Hsp90 associates with Cdk4 not with D-type cyclins. Inactivation of Cdc37/Hsp90 function by geldanamycin decreases the half-life of newly synthesized Cdk4, indicating that Cdc37/Hsp90 plays an important role in Cdk4 stabilization (Stepanova et al., 1996).

1.10.1 Hsp90 –independent activities of Cdc37

Most studies have shown Cdc37 working in co-operation with Hsp90. However, Cdc37 also displays Hsp90-independent activities. The combination of mutations in both Hsp90 and *cdc37* genes strongly affect the activity of sevenless receptor protein in *Drosophila* suggesting that Hsp90 and Cdc37 fulfill partially redundant functions (Cutforth and Rubin, 1994). In budding yeast, *MPS1* encodes an essential kinase required for spindle pole body duplication and for mitotic spindle assembly. Over expression of Cdc37 can suppress *mps1-1* temperature-sensitive growth. Although Mps1 protein levels are unaffected by the *cdc37-1* mutation, its kinase activity is markedly reduced. (Schutz et al., 1997). Expression of v-src is toxic to budding yeast, because it perturbs the formation of mitotic spindle in yeast. Mutations in either *CDC37* or the yeast Hsp90 genes lead to a decrease in v-src activity and suppress its toxicity, but the alteration in phosphorylation activity of v-src is different depending on which chaperone is defective (Kimura et al., 1997). Increased protein level of Cdc37 can partially compensate for a mutation in the Hsp90 gene to maintain activity of the heterologously expressed v-src (Kimura et al., 1997).

The protein kinase binding domain of Cdc37 mutant is sufficient for yeast cell viability and permits efficient signaling through the yeast MAP kinase-signaling pathway in budding yeast (Lee et al., 2002). Stil1, a Hsp90 co-chaperone, is essential in establishing "intermediate" complex containing the client polypeptide and Hsp90, which acts in recruiting Hsp90 to client polypeptide in the early stage of protein kinase maturation (Lee et al., 2002). Overexpression of *CDC37* can suppress a defect in v-src folding in yeast deleted for co-chaperone Sti1, which normally recruits Hsp90 to clients. Expression of Hsp90-binding site deletion *CDC37* truncation mutants can stabilize v-src activity and led to some folding in both *△sti1* and *△ hsc82* strains (Lee et al., 2002). These data suggest that Cdc37 can function independently of Hsp90.

1.10.2 Complexes with other Hsp90 co-chaperones

In addition to interaction with Hsp90, a number of other co-chaperones have been shown to interact with Cdc37 by genetic and biochemical methods. YDJ1, a budding yeast homologue to co-chaperone Hsp40 in mammalian cells, has been shown to interact with Cdc37 through synthetic lethal screen (Mort-Bontemps-Soret et al., 2002). Purified recombinant Sti1 protein, the *S.cerevisiae* homologue of mammalian Hop, can interact with Cdc37 protein in the absence of Hsp90. Furthermore the combination of *cdc37* and *sti1* mutations is synthetically lethal, suggesting that Cdc37 and Sti1 may contribute to important functions in yeast (Abbas-Terki et al., 2002). Overexpression of Cdc37 can restore the v-src activity in $\triangle$*sti1* strain in budding yeast. Yeast Cdc37 can interact biochemically with co-chaperones such as p23, FKBP52, Cyp40 (immunophilin) (Hartson et al., 2000) and Cpr7 (an immunophilin) (Abbas-Terki et al., 2002). Cdc37 may enter the chaperone-dependent folding pathway via interaction with other co-chaperones.

Cdc37 appears to compete for Hsp90 binding with TPR binding domain co-chaperone Cpr6/Cyp40 (Siligardi et al., 2002) and Hop (Silverstein et al., 1998) which form an alternative range of hetrocomplexes with Hsp90. Loss of function of one chaperone in the complex can result in the client being taken up by other co-chaperones. For example, treatment with geldanamycin will disrupt the binding between client protein such as Cdk9 and Hsp90-Cdc37 complexes (O'Keeffe et al., 2000), which are found to form complex with chaperone Hsp70.

1.10.3 Non-kinase client proteins of Cdc37

The substrates of Cdc37 are not solely the kinases. In yeast, heterologously expressed human androgen receptor (AR) requires Cdc37 function for full hormone-dependent transactivation (Fliss et al., 1997). In *cdc37ts* mutants, hormone-dependent transactivation by androgen receptors was defective at both permissive and restrictive temperatures, although glucocorticoid receptor was mildly defective only at the restrictive temperature (Fliss et al., 1997). Cdc37 protein binds to the androgen receptor through its ligand-binding domain, although it does not bind to the closely related glucocorticoid receptor (Rao et al., 2001). Hsp90 has been shown to associate with the reverse transcriptase (RT) of the duck hepadnavirus and is required for its functions. Cdc37 was found to interact specifically with RT (Wang et al., 2002). Although RT is not a kinase, it is structurally related to the kinase Raf-1 (Wang et al., 2002). A Cdc37 mutant defective in Hsp90 binding domain, could interact specifically with the RT, indicating that Cdc37 can bind the RT independently of Hsp90. It suggests that p50 can function as a cellular cofactor for the hepadnavirus RT by mediating the interaction between the RT and Hsp90.

1.11 Cdc37 localization

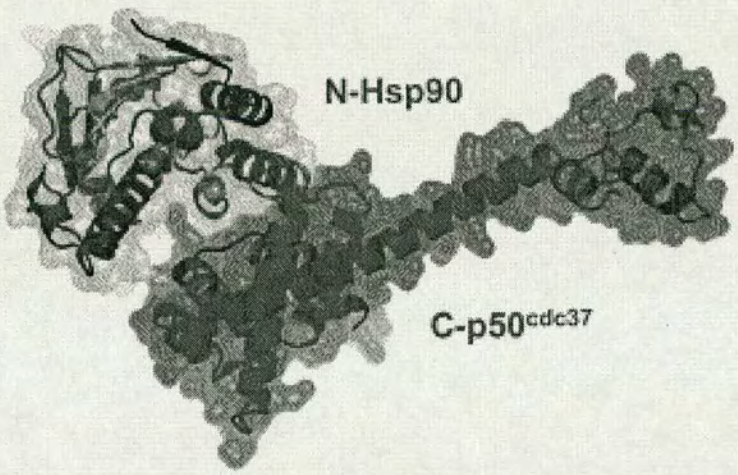
The mRNA levels of Cdc37 do not fluctuate during cell cycle in *S. cerevisiae* (Gerber et al., 1995; Turnbull et al., 2006). Subcellular localization studies of Cdc37 in *S. pombe* (Tatebe and Shiozaki, 2003; Turnbull et al., 2006) show Cdc37 protein is largely cytoplasmic, being diffuse throughout this region. In *Drosophila* cells, antibodies to Cdc37 stain the perinuclear region and give a punctate staining in the

cytoplasm (Cutforth and Rubin, 1994; Lange et al., 2002). Cdc37 was also detected to colocalize with the kinase Aurora B on the spindle microtubules and midbody during mitosis in *Drosophila* spermatocytes (Lange et al., 2002). Abolishing function of Cdc37 by RNAi in Drosophila results in cytokinesis failure (Lange et al., 2002).

1.12 Interaction between Cdc37 and Hsp90

A single Cdc37/Hsp90 complex is formed *in vitro* when the two proteins are mixed at 1:1. It suggests that Cdc37 binds to Hsp90 as a dimer (Zhang et al., 2004). The interaction of either full length or truncated variants of human Hsp90 with Cdc37 which was analysed by size-exclusion chromatography (SEC) shows that human Cdc37 can bind both N-terminal domain and the linker region of Hsp90. The complex between Hsp90 and Cdc37 was purified and subjected to limited proteolysis. The binding interface was identified as the stable 16 kDa unit comprising amino acids 147 to 276 of Cdc37 and 4 to 261 of Hsp90 (Zhang et al., 2004). A complex between N-terminal truncated yeast Hsp90 and C-terminal truncated human Cdc37 has been crystallised. The region of amino acids between 164-170 and 204-208 of Human Cdc37 forms a hydrophobic patch which interacts with the N-terminal region of yeast Hsp90 (Figure 1.5) (Roe et al., 2004).

Figure 1.5 Interaction of yeast Hsp90 and Human Cdc37 determined by X-ray Crystallography (Roe et al., 2004)



Amino acids 164-170 and 204-208 of human Cdc37 were seen to form a hydrophobic patch that interacts with the N-terminal region of yeast Hsp90

1.13 Introduction to *S. pombe*

1.13.1 Overview

The fission yeast *Schizosaccharomyces pombe* is a unicellular ascomycete fungus. *S. pombe* cells are rod-shaped, and grow by apical extension. It is only distantly related to the budding yeast *Saccharomyces cerevisiae* (Sipiczki, 2000). Its genome size is 13.8 Mb, and the DNA is contained on three chromosomes of 5.7, 4.6 and 3.5 Mb. Sequencing of the genome is now complete and data can be accessed through the Sanger Centre website under http://www.sanger.ac.uk/Projects/S_pombe/

1.13.2 *S .pombe* life cycle

If *S. pombe* cells are provided with sufficient nutrients, they enter the mitotic cell cycle, which will be described in more detail in the next section. *S. pombe* cells consists of two mating types, called h^+ and h^-. When deprived of nutrients, if cells of only one mating-type are present in the culture, they will exit the mitotic cell cycle and enter stationary G1 phase. If cells of both mating types are present in the same culture, they will accumulate in G1, conjugate and form a zygote. Zygotes undergo meiosis and sporulation by further nutrient deprivation. A zygotic ascus generates four spores, which will return to the growth cycle when nutritional conditions improve. If zygotes are re-supplied with nutrients before the initiation of meiosis, they will enter the mitotic cell-cycle (Figure 1.6).

Figure 1.6 A diagram of *S. pombe* life cycle

Under nutrient deprivation, hapoid cells exit the mitotic cell cycle, arresting in stationary phase or undergo conjugation to form diploid zygotes. The continuation of nutrient starvation of cells causes their entry into meiosis, resulting in sporulation producing an ascus that contains four spores. If nutrients are provided, the spores undergo outgrowth and resume the mitotic cell cycle (MacNeill et al., 1991).

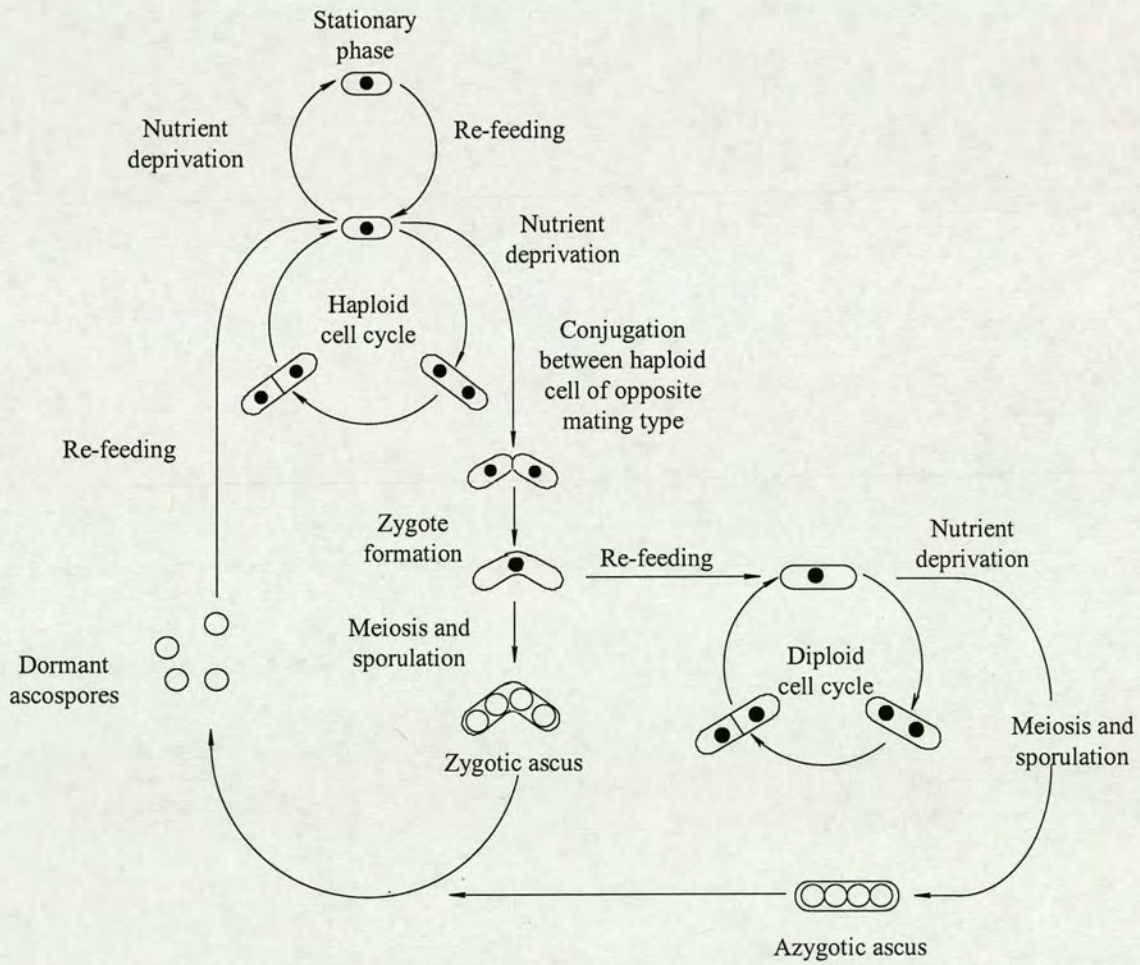
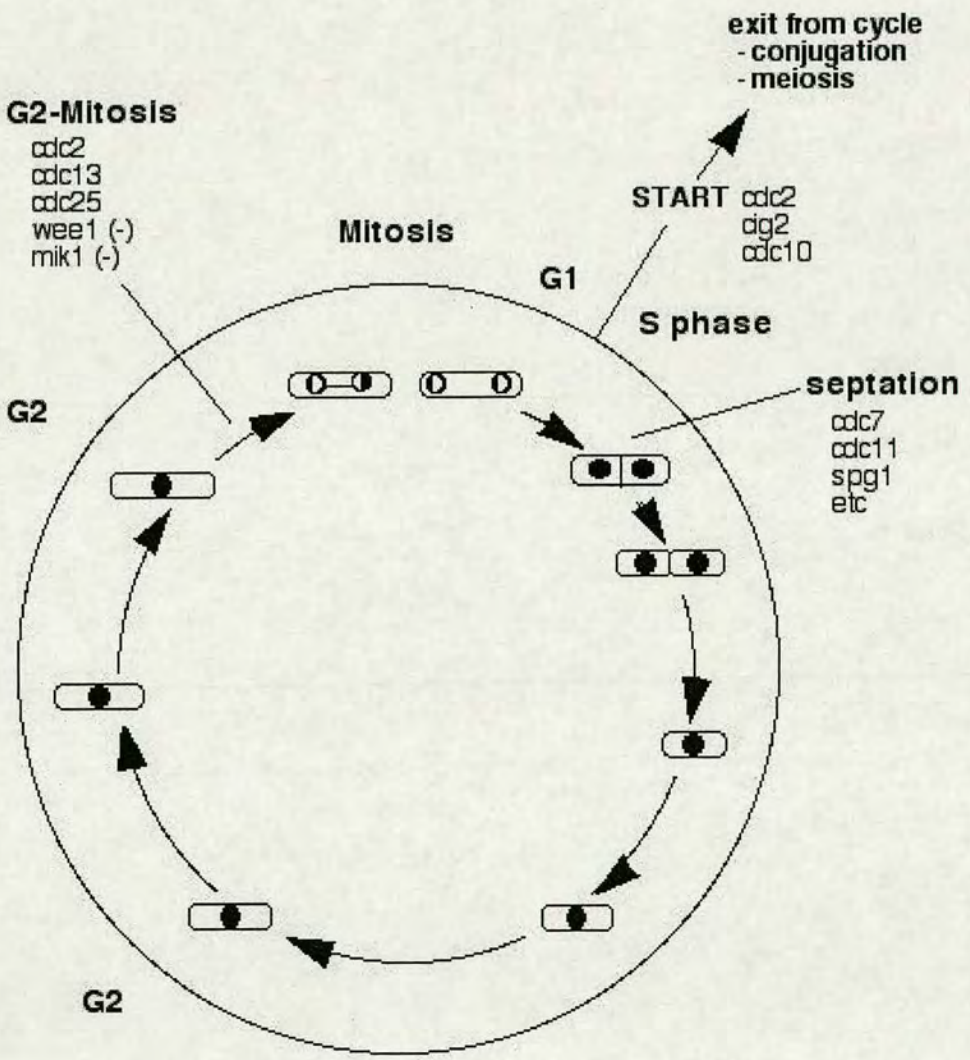
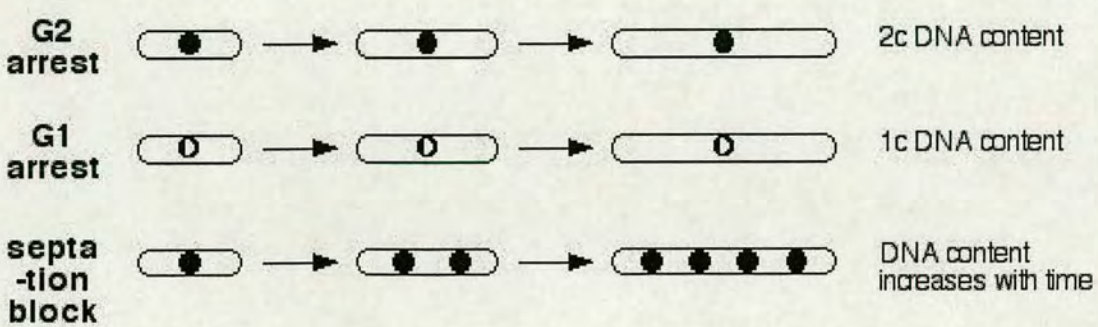


Figure 1.7 *S. pombe* cell cycle *S. pombe* possesses a typically eukaryotic cell cycle with distinct G1, S, G2 and M phases. In *S. pombe*, Cdc2 is the main Cdk that promotes the passages between G1/S phase and G2/mitosis. The cyclin partner proteins which appear periodically and rapidly degraded modulate their association with the Cdk and regulate the progression of the cell cycle.





1.13.3 *S. pombe* cell cycle

The mode of growth of fission yeast cell by length extension allows the stage of a cell within the cell cycle to be determined by measuring its cell length. *S. pombe* possesses a typically eukaryotic cell cycle with distinct G1, S, G2 and M phases (MacNeill et al., 1991). M, G1 and S phase each occupy ~10% of one cell cycle and G2 accounts for the remaining 70%. In G1 and G2, the cells undergo a period of growth. In S phase the genetic material is replicated and in mitosis, genetic material is segregated, followed by cleavage to generate two daughter cells (Figure 1.7).

There are two major control points in mitotic cell cycle of *S. pombe*: in late G1 before the initiation of S phase, and in late G2 before the initiation of mitosis. The point in G1 when cells become committed to the mitotic cell cycle termed Start (Nurse and Bissett, 1981). Passage of Start and entry into S phase are dependent on prior completion of mitosis and cells reaching a certain cell size (Nurse, 1975). The control point in late G2 determines the time of entry into mitosis. Passage of this G2/M control point requires that the cells reach a critical size and repair of any DNA damage that may have occurred (Humphrey, 2000).

Entry into each phase of the cell cycle is regulated by a series of protein kinases. Cyclin dependent kinases (Cdks) play an essential role in the cell cycle control. To enter S and M phase, the Cdk must be activated by association with an appropriate cyclin partner and phosphorylation or desphophorylation at specific residues.

Inactivation of the Cdk is induced when a failure in the cell activates a cell cycle checkpoint. In *S. pombe*, Cdc2 is the main Cdk that promotes the passages between G1/S phase and G2/mitosis. The cyclin partner proteins which appear periodically and rapidly degraded modulate their association with the Cdk and regulate the progression of the cell cycle. In *S. pombe*, two principal cyclin partners, Cdc13 and Cig2. Cdc13 associate with Cdc2 and regulate the G2/mitosis passage(Moser and Russell, 2000) And Cig2 is requred for regulation of G1/S passage (Bueno and Russell, 1993).

1.13.4 Cdc2 function in *S. pombe* cell cycle

In *S. pombe*, Cdc2 protein levels do not fluctuate throughout the cell cycle (Alfa et al., 1989). Cdc2 is the Cdk which is essential for the transitions between G2/M and G1/S phase. Regulation of cell cycle progression by Cdc2 is by associating with specific cyclin partners modulating its activity at different stages. Before the G2/M transition, Cdc2 is phosphorylated on threonine 167 by Cdk-activating kinases to promote cyclin association and on tyrosine 15 by Wee1 to keep the Cdk inactive during interphase (Den Haese et al., 1995). In late G1, phosphorylation on threonine 167 allows Cdc2 to associate with the B-type cyclin, Cdc13 (Alfa et al., 1989; Sohrmann et al., 1998). Cdc13 accumulates through interphase and is then degraded at the metaphase to anaphase transition (Alfa et al., 1989). To initiate mitosis, the complex of Cdc2-Cdc13 is activated by dephosphorylation on tyrosine 15 by Cdc25 (Nurse et al., 1976). The complex of Cdc2 and Cdc13 (known as M-phase promoting factor, MPF) is essential to initiate mitosis (Alfa et al., 1989).

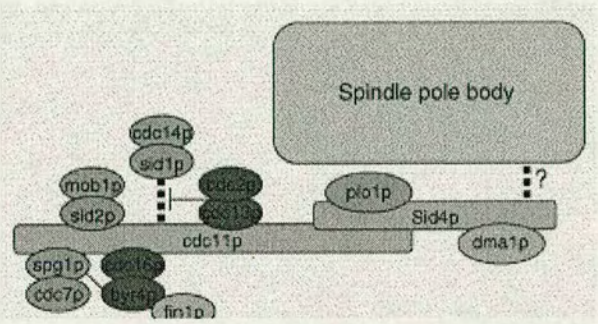
1.13.5 SIN pathway in late mitosis

S. pombe cells grow by elongation at their tips. The cell divides by formation of a septum in the centre of the cell. The septum is cleaved, producing two daughter cells of equal size. The *S. pombe* septation initiation network (SIN) is an important transduction pathway which synthesises the septum and brings the cell into cytokinesis (Simanis, 2003). Several protein kinases and a GTPase are identified as the components of SIN (Table 1.2). Spg1 encodes a GTPase which plays an important role in mediating SIN pathway. In GTP-bound form, Spg1 binds to the protein kinase Cdc7 (Sohrmann et al., 1998). Two protein kinases Sid1 and Sid2 and their subunits Cdc14 and Mob1 are required for the signal transduction (Guertin et al., 2000; Salimova et al., 2000). Cdc16 and Byr4 act as the GAP, which interacts with Spg1 and activates its activity (Jwa and Song, 1998). Cdc11 interacts with Sid4 and both localize to the spindle pole body at all stages of the cell cycle (Krapp et al., 2004) (Figure 1.9). It acts as the scaffold protein and is required for all the known SIN components to localize to the SPB. Polo-family kinase Plo1 is a regulator of SIN pathway, which is recruited to the SPB at the beginning of mitosis (Mulvihill et al., 1999a). Loss of function of SIN components *mob1*, *sid2* causes the elongated and multinucleated cells because cells can grow to past S phase and M phase in absent of cytokinesis. In contrast, overexpression of SIN components such as *spg1*, *cdc7,* or *plo1* results in multiple septa without cell cleavage.

Table 1.2 The components of SIN pathway in *S. pombe*

Genes	Descriptions
spg1	GTPase
cdc7	Protein kinase
sid1	Protein kinase
sid4	Scaffold
sid2	Protein kinase
cdc11	Scaffold
cdc16	GAP
byr4	GAP scaffold
plo1	Protein kinase

Figure 1.8 The SIN components at the SPB in *S. pombe* (Krapp et al., 2004)



1.14 This work

The *S. pombe cdc37* gene in *S. pombe* was isolated by PCR amplification of cosmid SPBC9B6 from genome (Westwood et al., 2004). It was found to be essential for cell viability. Depletion of Cdc37 results in a mixture of phenotypes, consistent with one or more kinases with cell cycle roles being compromised at low Cdc37 levels (Westwood et al., 2004). A mutant *cdc37* allele was also identified as during a genetic screen in fission yeast to identify novel components of the Spc1 SAPK cascade (Tatebe and Shiozaki, 2003). Overexpression of Wis1 MAPKK results in a lethal phenotype, because Spc1 MAPK activation was deregulated (Tatebe and Shiozaki, 2003). The *sws1* mutation was identified as a novel factor to rescue the lethal phenotype of Wis1 overexpression in Δ*atf1* strains (Tatebe and Shiozaki, 2003). A plasmid containing the $cdc37^{+}$ gene was obtained from the *S. pombe* genomic library by its ability to complement the *sws1-681* ts phenotype. The sequence result of *sws1* mutant shows a single amino acid mutation happens in *cdc37* gene.

The protein level of Spc1 in the *cdc37* mutant is reduced. And stress-induced phosphorylation of Spc1 by Wis1 is significantly reduced, which means that the interaction of Spc1 with Wis1 MAPKK is compromised in the *cdc37* mutant. Moreover, Cdc37 physically interacts with Spc1 in vivo. All these results indicate that Spc1 is a client for the Cdc37 chaperone. Cdc37 may be required for stabilizing the Spc1 protein and maintaining Spc1 in a properly folded state competent for the interaction with Wis1 MAPKK (Tatebe and Shiozaki, 2003). Although Cdc37 is often identified in Hsp90 complexes with kinases and is proposed to be a Hsp90

co-chaperone (Grammatikakis et al., 1999b; Stepanova et al., 1996), no apparent defect in the stability and activation of Spc1 MAPK was observed in the *swo1-26* mutant, suggesting that the Hsp90 function is not required for Spc1 function (Tatebe and Shiozaki, 2003).

Physical interaction between *S. pombe* Cdc37 and Hsp90 was only identified in a high molecular complex (Turnbull et al., 2005). Expression of only the N-terminal domain of *S. pombe* Cdc37, lacking the likely Hsp90-binding domain, can sustain cellular viability. It means that Cdc37 does interact with Hsp90 in *S. pombe* although the Hsp90-binding domain is not essential for cellular viability.

Cytological observation on several *cdc37ts* mutants reveals that at the restrictive temperature, *cdc37ts* cells were arrested during the cell cycle in G2 phase. Cdc2 activity in *cdc37ts* mutants is reduced because of the inability of Cdc2 to maintain a stable complex with the cyclin Cdc13. (Turnbull et al., 2006). In contrast to Cdc28 in *S. cerevisiae,* reduced Cdc2 activity in cdc37ts mutants is not the result of lower Cdc2 protein levels nor because Cdc2 aggregates into insoluble complexes(Farrell and Morgan, 2000; Turnbull et al., 2006). Both genetic and biochemical interactions between Cdc2 and Cdc37 were detected. Mutants containing temperature-sensitive mutant alleles for both Cdc2 and Cdc37 are synthetically lethal and Cdc2 co-immunoprecipitates with Cdc37. Cdc2 may be a client of Cdc37 that relies on this molecular chaperone to promote its activation by aiding in the assembly of complexes with Cdc13 (Turnbull et al., 2006).

Only the protein kinases Spc1 and Cdc2 had been identified as Cdc37 client proteins in *S. pombe*. Little is known about more client proteins of molecular chaperone Cdc37. In the work described in this thesis, a synthetic lethal genetic screen was carried out to find more client proteins. Candidate genes were

characterized to understand the interaction with Cdc37.

2. Materials and Methods

2.1 General

2.1.1 Chemicals and Enzymes

Chemicals were purchased generally from Sigma or Fisher unless otherwise stated. Enzymes which were used in this work were purchased from NEB or Roche unless otherwise stated. Agar, tryptone and yeast extract were purchased from Difco.

2.1.2 Commonly used reagents

TE (10x), pH 8.0	100 mM Tris-HCl, 10 mM EDTA, pH8.0
PBS (1x)	11.5 g/l Na_2HPO_4, 2.96 g/l NaH_2PO_4, 5.84 g/l NaCl
TAE (50x)	242 g/l Tris, 37.2 g/l EDTA, 57.1 ml/l acetic acid
Protein running buffer (5x)	15.1 g/l Tris, 94 g/l glycine, 5 g/l SDS
5xSDS loading buffer	250mM Tris-HCl, pH6.8, 10% (w/v) SDS, 0.5% (w/v) Bromophenol Blue, 50% (w/v) glycerol
Western blot transfer Buffer	18.8 g/l glycine, 3 g/l Tris, 0.1% (w/v) SDS, 20% (v/v) methanol
PBST	1xPBS, 0.1% (v/v) Tween-100

2.2 Plasmids

2.2.1 Introduction to pREP vectors

The pREP vectors with thiamine-regulatable *nmt* promoter (Maundrell, 1990) are commonly used for *S. pombe* gene expression. Sub-cloning the original version of nmt promoter into a *S. pombe* shuttle vector made the pREP3 vector. Two attenuated versions (pREP41 and pREP81) with reduced activity were constructed through mutating bases of TATA box (Basi et al., 1993). pREPX series were made by altering ATG codon in the original polylinker sequence (Forsburg, 1993).

Table 2.1 pREP vectors used in this work

Name	**Description**
pREP1	Strong promoter vector with *LEU2* marker
pREP41	Weaker promoter vector with *LEU2* marker
pREP4X	Strong promoter vector with *ura4* marker
pREP81	Weakest promoter vector with *LEU2* marker
pREP82	Weakest promoter vector with *ura4* marker

Table 2.2 Plasmids used in this work

Name	Reference	Description
pREP81-cdc37	(Westwood et al., 2004)	Contains full length of *cdc37*
pREP82-cdc37	(Westwood et al., 2004)	Contains full length of *cdc37*
pREP41-cdc7	(Fankhauser and Simanis, 1994)	3.6kb BamHI fragment of full length of *cdc7*
pREP4x-nak1	(Leonhard and Nurse, 2005)	Full length of *nak1*, pREP4x
pWF48	(Samejima et al., 1998)	Kinase domain of *wis4*, pREP1
pWF96	(Samejima et al., 1998)	Full length of *wis4*, pREP1
pSP1-msc1-HA	(Ahmed et al., 2004)	7.4kb SmaI-SacII fragment of full length of *msc1*

2.3 Bacterial Methods

2.3.1 Bacterial strains

The *E. coli* strain DH5α was used for propagation of plasmids and molecular cloning and the strain JM109 was used for DNA library amplification.

2.3.2 Bacterial medium

LB: 10 g/l tryptone
5 g/l yeast extract
5 g/l NaCl
For solid medium, agar was supplied at the final concentration of 2%

Table 2.3 Antibiotics

Antibiotic	Working concentration	Stock solution
Ampicillin	100 μg/ml	100 mg/ml in H_2O
Kanamycin	10 μg/ml	10 mg/ml in H_2O

2.3.3 Extraction of plasmid DNA from *E. coli*

Small scale plasmids preparations were carried out with the Qiagen Spin Miniprep kit, following the manufacturer's instructions. Medium scale plasmids were purified by Qiagen filter midi kit, following the manufacturer's protocol.

2.3.4 Preparing competent *E. coli* cells for transformation by electroporation

A preculture was set up by picking single DH5α or JM109 colonies from plate and inoculated in 3 ml LB liquid medium, shaking overnight at 37℃. 1ml preculture was added into 200 ml LB, incubating for 2-3 hours. When the culture reached OD_{600}= 0.4, cells were incubated on ice for 30 minutes. The cells were spun down for 15 minutes at 3000 rpm, and resuspended in 25 ml of chilled 10% glycerol twice. Finally, the cells were spun down at 3000 rpm for 15 minutes at 4℃. The pellet was resuspended and distributed into 50 μl aliquots, stored at -80℃.

2.3.5 Transformation of plasmid DNA into *E. coli* by electroporation

50 μl aliquots of cells were placed on ice to thaw. The cells were mixed with the same volume of ice cold 10% glycerol. 2 μg DNA was added to the cells in the cold cuvette. The mixture was pulsed at 1.8 kV, 200 Ω, 25 μF. 900 μl LB was added into the cuvette immediately after the pulse. Cells were transferred into 10 ml tube and incubated at 37℃ for 45 minutes to 1 hour. 0.1 ml of transformed cells was spread onto appropriate medium to grow up for 1 day until colonies were formed.

2.4 Fission yeast methods

2.4.1 Strains

Table 2.4 Strains used in this work are listed

Name	Genotype	Source
ED0665	*leu1-32 ura4-D18 ade6-M210 h⁻*	Lab stock
ED0666	*leu1-32 ura4-D18 ade6-M210 h⁺*	Lab stock
ED0862	*leu1-32 ura4-D18 h⁻*	Lab stock
ED1023	*rad27::ura4⁺ leu1-32 ura4-D18 h⁻*	Lab stock
ED1181	*wis4::ura4⁺ leu1-32 ura4-D18 h⁻*	Lab stock
ED1334	*win1::LEU2-32 ura4-D18 h⁺*	Lab stock
ED1428	*wis1::ura4⁺ leu1-32 ura4-D18 h⁺*	Lab stock
ED1538	*cdc37-681 leu1-32 ura4-D18 h⁻*	(Tatebe and Shiozaki, 2003)
ED1560	*swo1-26 leu1-32 h⁺*	Lab stock
ED1565	*cdc37-13 leu1-32 ura4-D18 h⁻*	Lab stock
ED1445	*cdc2-33 leu1-32 ura4-D18 h⁻*	Lab stock
ED1566	*cdc37-184 leu1-32 ura4-D18 h⁻*	Lab stock
ED1577	*msc1::kan^R leu1-32 ura4-D18 ade6-210 h⁻*	(Ahmed et al., 2004)
ED1584	*orb3-167 leu1-32 ura4-D18 ade6-M210 h⁻*	(Leonhard and Nurse, 2005)
ED1585	*orb3-35/2 ura4-D18 ade6-M210 h⁻*	(Leonhard and Nurse, 2005)
ED1586	*cdc37-681 leu1-32 ura4-D18 h⁺*	Lab stock
ED1587	*cdc7-24 leu1-32 h⁻*	(Fankhauser and Simanis, 1994)
ED1588	*cdc37-681 cdc7::HA leu1-32 ura4-D18 h⁻*	Lab stock
ED1589	*cdc37-184 cdc7::HA leu1-32 ura4-D18 h⁻*	Lab stock
ED1590	*cdc37-13 cdc7::HA leu1-32 ura4-D18 h⁻*	Lab stock
ED1595	*cdc37-681 leu1-32 ura4-D18:pREP82-cdc37 h⁻*	Lab stock
ED1596	*cdc2-33 leu1-32 cdc7-24::HA ura4-D18 h⁻*	Lab stock

ED1597	*cdc7::HA leu1-32 h⁻*	(Cerutti and Simanis, 1999)
ED1598	*cdc7::GFP leu1-32 h⁻*	(Cerutti and Simanis, 1999)
ED1599	*cdc7::GFP leu1-32 h⁺*	(Cerutti and Simanis, 1999)
ED1600	*pcp1-mcherry mad1:GFP leu1-32 ura4-D18 ade6-M210h⁻*	(Jin et al., 2002)
ED1591	*cdc7-GFP pcp1-mcherry leu1-32 ura4-D18h⁻*	Lab stock
ED1592	*cdc37-184cdc7-GFP pcp1-mcherry leu1-32 ura4-D18h⁻*	Lab stock
ED1593	*cdc37-681cdc7-GFP pcp1-mcherry leu1-32 ura4-D18h⁻*	Lab stock
ED1594	*cdc37-184 leu1-32 ura4-D18h⁻*	Lab stock

2.4.2 Yeast medium

YE

S. pombe strains were grown on YE. It contains all the components necessary for good growth and inhibits conjugation and sporulation.

YE: 5 g/l yeast extract
30 g/l glucose
120 mg/l adenine, histidine, leucine, lysine hydrochloride and uracil

EMMG (Edinburgh Minimal Medium Glutamate)

EMMG is free of thiamine and was used for vegetative growth.

EMMG: 3 g/l Potassium hydrogen phthalate
2.2 g/l Sodium phosphate dibasic
20 g/l glucose
2% salts (50x stock)

0.1% vitamins (1000x stock)

0. 01% minerals (10,000x stock)

1 g/l sodium glutamate

Salts (50x stock):

52.5 g/l $MgCl_2 \cdot 6H_2O$

0.735 mg/l $CaCl_2 \cdot 2H_2O$

50 g/l KCl

2 g/l $Na_2SO_4 \cdot 2H_2O$

Vitamins (1000x):

1 g/l pathothenic acid

10 g/l nicotinic acid

10 g/l myo-inositol

10 mg/l biotin

Minerals (10,000x):

5 g/l boric acid

4 g/l $MnSO_4 \cdot H_2O$

4 g/l $ZnSO_4 \cdot 7H_2O$

2 g/l $FeCl_2 \cdot 4H_2O$

0.4 g/l molybdic acid

1 g/l KI

0.4 g/l $CuSO_4 \cdot 5H_2O$

10 g/l citric acid

SPA

SPA medium starves the cells of nitrogen and is used to stimulate meiosis and sporulation.

SPA:

1% (w/v) glucose

7.3 mM KH_2PO4

0.1% vitamins (1000x stock)

45 mg/l adenine, histidine, leucine, lysine

hydrochloride and uracil 2% agar

Table 2.5 Amino acid supplement for EMMG

Amino Acid	stock concentration	Working concentration
Adenine	3.75g/l	112.5mg/l
Leucine	7.5g/l	112.5mg/l
Uracil	3.75g/l	112.5mg/l
Histidine	7.5g/l	112.5mg/l

5FOA (Toronto Research Chemical)

Using 5-Fluoro-orotic Acid (5-FOA) for the selection of yeast is a commonly used genetic screening method. 5-FOA is converted to a toxic compound in yeast strains containing the functional *S. pombe ura4*$^+$ gene coding for orotine-5'-monophosphate decarboxylase. 5FOA was added into melted EMMG agar medium at 55℃ at the concentration of 1mg/ml.

G418 (Stratagene)

G418 was added into melted YE agar at a final concentration of 1g/l at 55℃.

FK506 (Calbiochem)

FK506 affects calcineurin signal transduction in fission yeast (Yabin et al, 2002). Medium containing FK506 usually inhibits *cdc7* mutants' growth. It was added into YE medium at a final concentration of 50 μg/ml.

Phloxin B

Phloxin B stains the dead cells of *S. pombe*. A number of dead cells in diploid colonies will be dyed dark pink. It was used to distinguish diploid and haploid cells.

Phloxin B was added at the concentration of 1.75 μg/ml.

ClonNAT (Werner BioAgents)

The working concentration is 100 μg/ml.

2.4.3 Waking up *S. pombe* strains

A small amount of cells were scraped from a frozen stock tube. Samples were streaked onto YE medium or EMMG medium plus supplements, incubated for 3 days. Single colonies were picked and tested for correct genotype, then incubated in medium for further use.

2.4.4 Storage of *S. pombe* strains

S. pombe strains were kept on plates at 4℃ for 1-2 months. For longer term storage, fresh cell suspension was mixed with the same volume of 50% glycerol in YE, kept in cryotube and stored at -70℃.

2.5 General DNA methods

2.5.1 DNA extraction by using phenol-chloroform

DNA solution was mixed with an equal volume of Tris equilibrated phenol, vortexed several times and, spun down at 12000 rpm for 5 minutes. The upper layer was transferred into a new 1.5 ml eppendorf tube. The supernant was treated with an equal volume of phenol-chloroform-isoamylalcohol (25:24:1) twice as described above.

2.5.2 DNA precipitation by isopropanol

An equal volume of chilled isopropanol was added to the DNA solution, mixed thoroughly and incubated at -70℃ for at least 1 hour. Then the mixture was centrifuged at 12000 rpm, 4℃ for 20 minutes, washed by 100% ethanol and 70% ethanol once. The pellet was dissolved in 25-50 μl TE.

2.5.3 Restriction digestion of DNA

Restriction enzyme digests of DNA were usually carried out in 20 μl reaction system. Analytical digests contained 0.5 μg DNA, 2 μl 10x reaction buffer, 1μl BSA (50x stock), 1-2 units enzyme. Large scale digests for sub-cloning, 40 μl reaction system contained 2-3 μg DNA, 4 μl 10x reaction buffer, 2 μl BSA (50x stock), 3-4 units enzyme. The mixture was incubated at the appropriate temperature for 1 hour. The result of the digestion was analyzed by agarose gel electrophoresis.

2.5.4 Phosphatase treatment of DNA ends

Calf intestinal alkaline phosphatase (CIP) was used to remove 5' phosphate ends of linear plasmid DNA after restriction digestion to reduce plasmid self ligation in subsequent ligation reaction. Typical treatment, 1μl CIP was mixed with 2μg digested plasmid DNA and 5μl 10x reaction buffer, incubated at 37℃ for 30 minutes. The phosphatased DNA was purified by Qingen QIA quick gel extraction kit.

2.5.5 Ligation of DNA fragments

Each ligation reaction carried out in the volume of 20 μl, containing 0.5 μg DNA

fragments with 1 µl T4 DNA ligase and 2 µl reaction buffer at 16℃ overnight. Vector and insert DNA were applied in an approximately 1:3 molar ratio.

2.5.6 Agarose gel electrophoresis

DNA samples were run in 0.8-1.0 % (w/v) agarose gels. Gels were prepared by melting the amount of agarose powder in 1xTAE and ethidium bromide was added at the final concentration of 0.1 µg/ml. Samples were mixed with 2x DNA loading buffer and run at a voltage of 70-80V for 2 hours. The size of separated fragments was compared with appropriate DNA molecular weight markers. DNA was visualized by UV lamp.

2.5.7 DNA amplification

The polymerase chain reaction was used to amplify DNA fragments. Taq polymerase (Sigma) was used for general applications. In case the amplified DNA used to be cloned into vector, Pfu (Roche) polymerase was used to minimize errors during the amplification.

An analytical 20 µl reaction contained:

DNA	100 ng
5' oligonucleotide (100 nmol)	1 µl
3' oligonucletide (100 nmol)	1 µl
10 x polymerase buffer (Sigma)	2 µl
dNTPs	0.4 µl
DNA polymerase	1 unit
dH_20	to 20µl

0.2 ml PCR tubes were used for the mixture, and the reactions were carried out in PCR machine (Flexigene).

PCR program:

Step 1: 95℃	1	minute
Step 2: 60℃	1	minute
Step 3: 72℃	1.5	minutes
	30 cycles	
Step 4: 72℃	7	minutes

2.5.8 DNA Sequencing

Plasmid DNA or PCR products were purified using gel extraction kit. Sequencing reactions were carried out with BigDye cycle sequencing kit (Applied Biosystem) in PRC machine. Then samples were run by university sequencing service on an ABI PRISM 377 DNA sequencer. The data was analyzed by the program Bio Editor on a PC.

Reaction mixture:

Template DNA:	200 ng
Primer:	3.2 μM
Terminator Ready Reaction mix	4 μl
dH_20	to 20 μl

Program:

Step1: 94℃	3	minute
Step2: 96℃	0.5	minute
Step3: 50℃	15	seconds
Step4: 60℃	4	minutes
30 cycles		
Step5: 60℃	1	minute

2.6 Genetic analyses

2.6.1 Procedure for crossing fission yeast strains

Small amounts of fresh growing colony of h^+ and h^- strains to be crossed were patched on SPA plates and mixed with a drop of water; dried briefly then incubated at 28℃ for 2-3 days.

2.6.2 Random Spore analysis

Samples were scraped from crosses, then suspended in 1ml H_2O, added 5 μl, 1:10 diluted Helix pomatia digestive juice (Sepracor). The enzyme is able to digest the asci walls and release the spores. The vagetative cells were killed by the enzyme.The suspension was incubated at 36℃ overnight. Spores were spun down at 13,000 rpm for 25 seconds, washed with 1ml water, resuspended in 1ml fresh water, and then diluted serially. 100μl aliquots were spread on the plates, and the plates were incubated at appropriate temperature until the colonies formed.

2.6.3 Tetrad analysis

Thin YE plates were poured and dried before dissection. A small amount of material from the cross was spread on the prepared plates with a drop of water at designated location. Asci were dissected by tetrad dissector, followed the manufacturer's instructions. Spore plates were incubated at 28℃ until the colonies formed.

2.7 *S. pombe* DNA manipulations

2.7.1 Transformation of plasmid DNA into *S. pombe* cells by electroporation

A toothpick of fresh cells was inoculated in 10 ml YE medium for 2 days at appropriate temperature. A small amount of preculture was inoculated into 200 ml fresh YE liquid medium and shaken overnight. When the culture had reached an OD_{600} of 0.3 to 0.4, cells were spun down and washed three times with 1.2 M ice-cold sorbitol. Cell pellet was resuspended in 1 ml ice-cold 1.2 M sorbitol at a final concentration of 1×10^9 cells/ ml. 0.2 ml cell suspension was mixed with 0.5 μg DNA and transferred into a sterile ice-cold cuvette. The cell suspension was pulsed at 2.5 kV, 200 Ω, 25 μF. 1.2 M ice-cold sorbitol was added into the cuvette immediately after the pulse. 0.2 ml of transformed cells was spread onto appropriate medium to incubate for 4 days until the colonies were formed.

2.7.2 Extraction of plasmid DNA from *S. pombe*

A patch of cells was inoculated in 10 ml selective liquid medium for 2 days. The culture was centrifuged at 7500 rpm for 5 minutes, and washed with 1 ml water. Cell pellet was gently resuspended in 1.5 ml Spheroplast buffer with Zymolyase-20T (2 mg/ml. The resuspended cells were incubated at 37℃ for 30 minutes to one and a half hours. Cells were centrifuged at 7500 rpm for 5 minutes and suspended in 0.3 ml TE, then 35 μl 10% SDS was added and incubated at 65℃ for 10 minutes. 100μl ice-cold 5M KOAc was added and incubated on ice for 20 minutes followed by centrifugation. The supernatant was mixed well with an equal volume of chilled 100 % isopropanol and put at -70 ℃ for 1 hour. The pellet was collected by centrifuged at 14,000 rpm for 20 minutes at 4℃ and resuspended in 20 μl water. 10μl sample can

was used to transform *E.coli.*

Spheroplast buffer: 50 mM citrate/phosphate buffer, pH5.6

1.2 M sorbitol

2.7.3 Mutagenesis of *S. pombe*

A haploid *cdc37*ts mutant ED1538 was transformed by pREP82 plasmid carrying a wild –type copy of cdc37 gene to create strain ED1595 (*cdc37-681 leu1-32 ura4-D18:pREP82-cdc37 h*$^-$). ED1595 was grown up in 200 ml supplemented EMMG liquid medium overnight. 10 ml cells were harvested at 3000 rpm for 5 minutes, washed once with TM buffer (50mM Tris-Maleate pH 6). The pellet was resuspended in TM buffer at a final concentration of 1.4 x 10^8 cells/ ml. 700µl cell suspension was mixed with 300µl 1mg/ml NMG in the same buffer and incubated at 30℃ for 30, 60, 90 minutes with occasional vortexing.100µl suspending cells was removed and diluted with 900µl TM buffer, then washed twice with 1ml of fresh EMMG medium. The pellet was resuspended in 1ml EMMG and incubated for 4 hours at 28℃. The samples were spread onto EMMG medium for counting the survivors after serial dilution. Glycerol was added to aliquots at the final concentration of 25%.The remainders were kept in -70℃ for screening.

2.8 Protein work

2.8.1 *S. pombe* small scale protein extraction by using Ribolyser

S. pombe cells were grown to OD_{600}=0.4 in YE. 50 ml of cells were spun down at 3000rpm for 5 minutes. Pellets were washed twice with 10 ml dH_2O. Cells were

drained in the eppendorf tube. To the pellet was added an equal volume of acid washed glass beads and 300µl denaturing extraction buffer. The tubes were placed on the Ribolyser machine and processed at 4.0 g for 20 seconds. The samples were heated for 5 minutes at 95℃, centrifuged and 12.5 µl supernant was removed for running a gel. For native protein extraction, a similar procedure was followed but using 1ml native extraction buffer. Samples were processed by Ribolyser.

Denatured buffer:

2 x SDS-PAGE loading buffer	300 µl
100 mM DTT	30 µl
1mM Pefabloc SC (Protease inhibitor)	3 µl
1mM LPC (Protease inhibitor)	1 µl

Native buffer:

100 µl 1 x complete inhibitor (Roche)

50 mM Tris-HCl

0.1mM Sodium orthovanadate

1mM DTT

60mM β-glycerophosphate

0.1mM EDTA

1mM PMSF

1% (v/v) NP-40

dH_2O to a total volume of 1 ml

2.8.2 SDS-polyacrylamide gel electrophoresis

Gels were poured using Mini-Protean III cell (bio-rad) for polyacrylamide gels. The upper stacking gels had acrylamide concentration of 5%, which helps the protein to

be concentrated for better resolution. The separating gels had the acrylamide concentrations from 12%-15% depending on the molecular weight of the target proteins. The gel system was assembled according to the manufacturer's instructions. 3ml of gel solution was mixed and poured for separating layer. A small amount of isopropanol was added to assure an even surface of separating gel during the polymerization. After separating gel was polymerized, 1 ml stacking gel was poured. The apparatus was fitted in the tank and filled with running buffer. Samples were mixed with 2x protein loading buffer and run at a voltage of 70-80V for 2 hours.

Table 2.6 SDS-PAGE gel component

	Stacking Gel 5%	Separating Gel 12%	Separating Gel 15%
1.5M Tris –HCL, pH8.8		2.5 ml	2.5ml
0.5M Tris –HCL, pH6.8	2.5ml		
30% Acrylamide solution with 2% bisacrylamide	1.33ml	4ml	5ml
10%(w/v) AMPS	70µl	70µl	70µl
TEMED	5µl	5µl	5µl
dH_2O	6.1ml	3.35ml	2.35ml
	10ml	10ml	10ml

2.8.3 Western blotting

The separating gel was washed in water for 5 minutes, and then placed into transfer buffer for 5 minutes. A PVDF membrane was dipped in methanol for 1 minute.

Whatman 3MM paper and membrane were soaked in 1x transfer buffer for 5 minutes. The blotting apparatus was assembled and run for 1 hour at 70-100 volts.

After transferring, membrane was dipped into 30 ml PBST with 4% milk powder on the shaker for blocking 1 hour at room temperature. The membrane was washed with 20ml PBST three times for 3x5 minutes. The membrane was placed into a small box and incubated with 10 ml of PBST plus primary antibody at 4℃ overnight with gentle shaking. The next day, the membrane was washed with PBST as described above and incubated with 10 ml of PBST plus secondary antibody at room temperature for 2 hours. It was washed with PBST for three times. The membrane was covered with ECL detection kit (Amersham) ,1:1 mixture of solution 1 and 2 and incubated for one minute. Excess of liquid was removed and the membrane was wrapped and placed in a film cassette. The blot was exposed to Hyperfilm ECL film (Amersham) for 30 seconds to 5 minutes and developed.

Table 2.7 Antibodies used in this work

Name	Description	Supplies
12AC5 Anti-HA	Monoclonal	Roche
Goat Anti-mouse	IgG HRP linked monoclonal	Amersham

2.8.4 Immunoprecipitation

20 mg of Protein A Sepharose CL-4B (Amersham) beads were weighed and hydrated with 1 ml of dH_2O for 10 minutes. Beads were spun down at 7500 rpm for 3 minutes and rinsed three times with 500 µl chilled lysis buffer. After final centrifugation,

beads were resuspended in 80 µl of lysis buffer. 20 µl of beads slurry was used for each IP. An aliquot of antibody was added to the bead suspension in a total volume of 100 µl lysis buffer. The tube was fixed on a rotating wheel and incubated for 1 hour at 4℃. The beads were washed with 500µl lysis buffer three times by centrifugation. The 100 µl native protein extract was mixed with the beads and incubated for 2 hours on a rotating wheel at 4℃. Beads were washed with 500 µl lysis buffer three times and transferred into a new tube. Half of sample was mixed with 30 µl of 2x SDS loading buffer and boiled for running on a gel. The remainder was kept in -80℃.

2.8.5 Assay of Cdc7 kinase activity

MBP (myelin basic protein) was used as the substrate for assaying Cdc7 kinase activity. $4x10^8$ cells were washed with 20 ml of water and resuspended in 100µl extraction buffer. Samples were processed with acid washed glass beads by Ribolyser machine. The beads were washed with 1 ml lysis buffer. The supernatant was transferred into a new tube.

Protein A Sepharose CL-4B beads were bound with an appropriate amount of antibody. 100µl native protein extract was mixed with the beads and incubated for 2 hours on a rotating wheel at 4℃. Beads were washed with 500 µl lysis buffer three times and transferred into a new tube. 20 µl of sample was mixed with 20 µl of 2x SDS loading buffer, boiled for running a gel and analysis by western blot. 10µl of beads was washed with the kinase buffer. For kinase assay, 10 µl of beads were incubated at 37℃ for 5 minutes and 10 µl kinase reaction buffer was added. After 15 minutes incubation at 37℃, the reaction was stopped by adding the same volume of 2x SDS loading buffer and boiled for 3 minutes. 10 µl of the sample was loaded on the gel, running, fixed and dried, autoradiographed.

Kinase buffer:

100 µl 1 x complete inhibitor

50 mM Tris-HCl

0.1mM Sodium orthovanadate

60 mM β-glycerophosphate, pH 7.5

15 mM EGTA

15 mM $MgCl_2$

1 mM PMSF

dH_2O to a total volume of 1 ml

Kinase reaction buffer

Kinase buffer

500 μg/ml MBP

20 μM ATP (Roche)

2 μCi [γ-^{32}P] ATP (10mCi/ml)(Amersham)

2.10 *S. pombe* microscopy

2.10.1 Cytological methods. For visualisation of the nuclear DNA, cells were washed and fixed in methanol at -20°C or in 3.7% (w/v) formaldehyde, mounted in antifade solution containing DAPI (4,6,diamidino-2-phenylindole) (10μg/ml) and examined under UV excitation on a Zeiss fluorescence microscope. Cell walls and septa were visualised by inclusion of Calcofluor (1ug/ml) (Sigma). Cdc7-GFP and Pcp1-mcherry were visualised using blue and green excitation filter sets respectively on an Intelligent Imaging Innovations Marianas system, which incorporates a Zeiss Axiovert microscope, CoolSnap CCD, and Slidebook software.

3 Screen for mutants showing synthetic lethality with *cdc37*

3.1 Introduction

3.1.1 General introduction

As described, Cdc37 has molecular chaperone activity and plays an important role in many cellular processes. A variety of techniques have been applied for identification of the possible clients of Cdc37.

Co-immunoprecipitation (Co-IP) is one of the most widely used immunochemical techniques. It is routinely used in the application to study protein/protein interactions. In the IP method, the Cdc37 protein from the cells was precipitated in an appropriate lysis buffer by means of an immune complex which includes the Cdc37 interacting-protein. Then Cdc37 protein can be identified by anti-Cdc37 antibody.

In recent years, Tandem Affinity Purification (TAP- purification) has been applied for identification of novel interactions. In this method, a protein of interest is fused to two affinity tags. The interacting partners along with the fusion protein are recovered by two specific affinity purification and elution steps. Cdc37 protein was found by TAP- purification to form a complex with MEKK1, MEKK3, TAK1 and TBK1 in human TNF-α/NF-κB signal transduction pathway (Bouwmeester et al 2004).

Suppressor analysis is a commonly used genetic tool to identify gene and protein interactions. Cdc37 had been identified as a multi-copy suppressor of *mps1-1* in

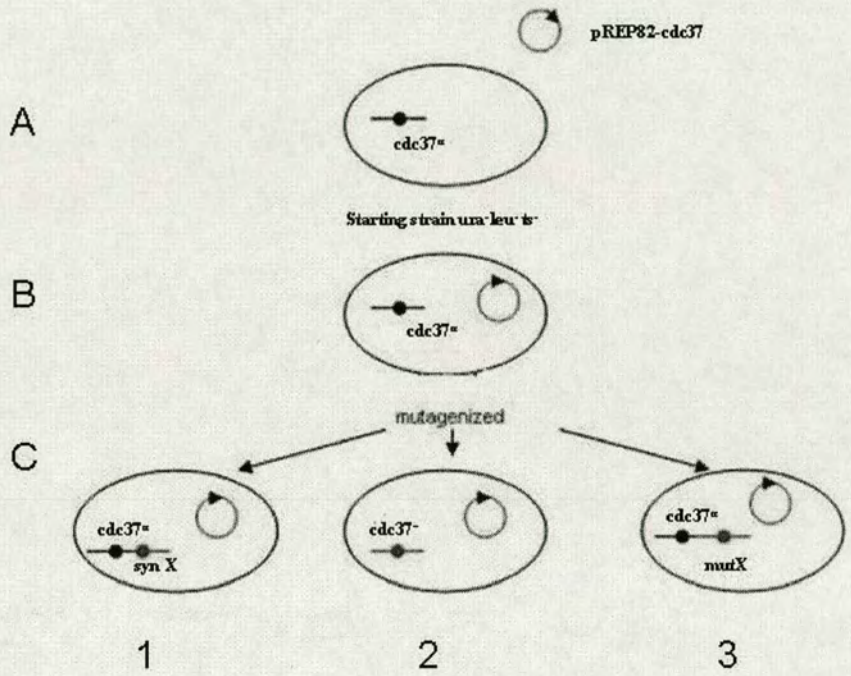
Saccharomyces cerevisiae by suppressor analysis (Schutz et al 1997). The observation strongly predicted that Mps1 might be a possible client of Cdc37. The interaction between Cdc37 and Mps1 was confirmed by molecular and cytological assays (Schutz et al., 1997).

3.1.2 Strategy

Synthetic lethal screen is a powerful tool for identifying gene interactions in the pathway of interest. Two mutations are considered synthetically lethal if in combination, they result in cell death. It can be used for seeking mutations at additional loci that enhance the phenotype caused by a particular mutation (Forsburg et al, 2001). Synthetic lethality can be caused by two genes acting in a parallel redundant pathway, or the two genes can act in the same pathway.

In this project, a temperature sensitive strain was used as the starting strain. *cdc37-681* was isolated as a single mutation in the position of amino acid 285 (Tatebe and Shiozaki, 2003). As a conditional mutant, *cdc37-681* is growing at the permissive temperature of 28 °C. Although cells are viable, the Cdc37 function is attenuated at this temperature. The strain was transformed with a plasmid pREP82-cdc37 expressing the wild type version of *cdc37*. The cells were mutagenized by chemicals and then screened at the permissive temperature. During the screening, *ura4*$^{+}$ marker in the plasmid can be used to select for the cells that have lost the plasmid. The *S. pombe ura4*$^{+}$ gene which encodes orotidine 5'-phosphate decarboxylase, results in cells sensitive to the toxic analogue 5-FOA (5'-

Figure 3.1: Identification of mutations that are synthetically lethal with *cdc37* in fission yeast.



A. to isolate synthetic mutants, the starting strain contains a *cdc37* temperature sensitive mutation and a plasmid expressing the wild-type version of the gene.

B. The cells are mutagenized and then screened for loss of the plasmid on medium containing 5-FOA or thiamine

C. (1) The desired mutants will not survive on the 5FOA, because they have a mutation that requires the presence of *cdc37*$^{+}$ for viability. (2) If the mutation occurs in *cdc37* itself and abolishes its function, the cells also need the plasmid for survival. The colonies would be 5-FOA sensitive. (3) If the new mutation happens in a second irrelevant gene, the cells can keep alive without the plasmid.

fluoro-orotic acid). So, cells which have lost the plasmid can be identified on 5-FOA plates (Figure 3.1). There are three possible cases after mutagenesis. One is that an additional mutation occurs in a gene that results in synthetic lethality. In this situation, the cells need the plasmid to provide Cdc37 function for its viability. The colonies would be 5-FOA sensitive and kept for future research. The other case is that the new mutation happens in a second irrelevant gene, the cells can keep alive without the plasmid. The colonies would be 5-FOA resistant and discarded. If the mutation occurs in *cdc37* itself and abolishes its function, the cells also need the plasmid for survival. The colonies would be 5-FOA sensitive and kept for future research.

3.2 Making the starting strain

To isolate synthetic lethal mutants efficiently, the selection of the starting strain is most important. There are two choices for two different screen strategies. One is that the starting strain has a null mutation in the original gene. Then after mutagenesis, the mutants which need the plasmid for survival have the mutations in synthetic lethal gene. In the second strategy, if a starting strain with a conditional mutation issued, the mutants which need the plasmid for survival will have the mutations either in the synthetic lethal gene or the original gene itself. In this project, *S. pombe cdc37* is an essential gene; deletion of *cdc37* will cause cells to stop growing (Westwood et al, 2003). The temperature sensitive mutant was therefore used for constructing starting strain.

For making the starting strain, a haploid *cdc37-681* mutant ED1538 (*cdc37-681 leu1-32 ura4-D18*) was transformed by the plasmid pREP82-cdc37 to generate ED1595. Rep82 has the thiamine-regulatable nmt promoter (Basi et al, 1993). For screening the synthetic lethal mutants after mutagenesis, the selection would be

carried on 5-FOA to score the mutants which lost the plasmid. Or it could also be carried on the media containing thiamine to identify the strain has the plasmid, but expression of *cdc37* has been shut down by the presence of thiamine. After incubation at 28 ℃ for 4 days on EMMG supplemented with leucine, colonies were picked for further testing.

3.3 Characterization of starting strains

The cell morphologies of *cdc37-681* with the pREP82-cdc37 named ED1595, *cdc37-681* ED1538 temperature sensitive strain, *cdc37* $^{+}$strain ED0862 were observed under the microscope after overnight incubation on EMMG plus leucine plates at the temperatures of 28 ℃ and 36 ℃. At 28 ℃, ED1595, ED1538 and *cdc37*$^{+}$ cells were normal in appearance. At 36 ℃, ED1595 and *cdc37*$^{+}$ cells were morphologically normal, while *cdc37* temperature sensitive mutant cells (ED1538) were elongated and characteristic of the cell division cycle defect (Figure 3.2). The results confirm that ED1595 had the mutation in *cdc37* gene and the plasmid carried the wild type version of *cdc37* gene can rescue the defect caused by the mutation.

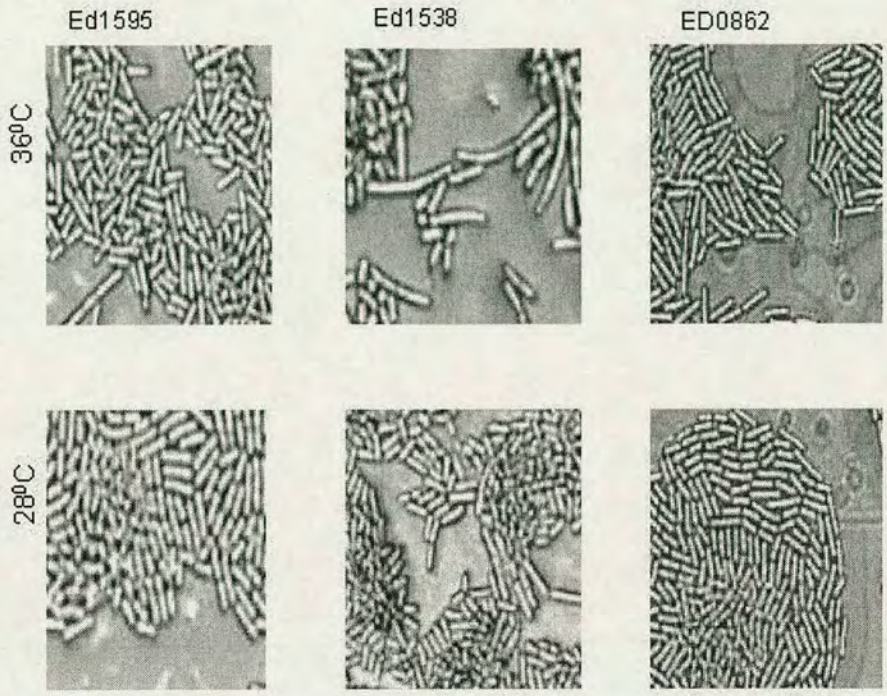
To test the selective markers, the transformants also were plated on EMMG plus leucine and uracil with or without 5-FOA at 28 ℃ after two days' incubation. The cells showed resistance in the presence of 5-FOA. It indicated that the ED1595 can survive at 28 ℃ without the plasmid carrying the *cdc37* gene. Because thiamine was an alternative selective agent considered for isolation of the mutants, the candidate transformants also proliferate on the EMMG plus leucine with or without thiamine at 28 ℃ after two days' incubation. Cells grew on both selective media. This suggested that cells are viable without *cdc37* expression from the plasmid. I wondered whether thiamine can effectively shut down the expression of exogenous *cdc37* gene. To test this, ED1595 was plated on media with and without thiamine at 36 ℃ for 2 days. The cells exhibit the same phenotype of *cdc37* ts mutants at the

high temperature, when thiamine was present. In contrast, the cells looked normal at the high temperature when thiamine was absence. The explanation for this is that thiamine negatively regulated the expression of Cdc37 protein from the plasmid. The cells cannot grow with the defect in *cdc37* gene at high temperature.

3.4 Mutagenesis of candidate strains

MNNG (N-Methyl-N'-Nitro-N-Nitrosoguanidine) was chosen to create the mutants as it works efficiently in *Saccharomyces cerevisiae* (Kohalmi and Kunz, 1988). Treatment of cells with *N*-methyl-*N'* -nitro-*N*-nitrosoguanidine (MNNG) produces mutagenic and carcinogenic lesions such as O^4-methylthymine in DNA. Two points that need to be thought about were the cell density of the suspension during the treatment of chemical and the time of the treatment. The two factors will affect the final kill rate of the mutagenesis. Studies on budding yeast shown the kill rate between 50%-90% is required to produce enough mutants in the surviving population. Higher kill rates result in an increased frequency of mutants with multiple mutations in the genome (Lawrence, 1991). Lower kill rates may not give rise to enough mutants in the viable cell population. The data were used to estimate the appropriate kill rate in *S. pombe.*

Figure 3.2 Phenotypes of ED1595, ED1538 and ED0862 at permissive and restrictive temperatures

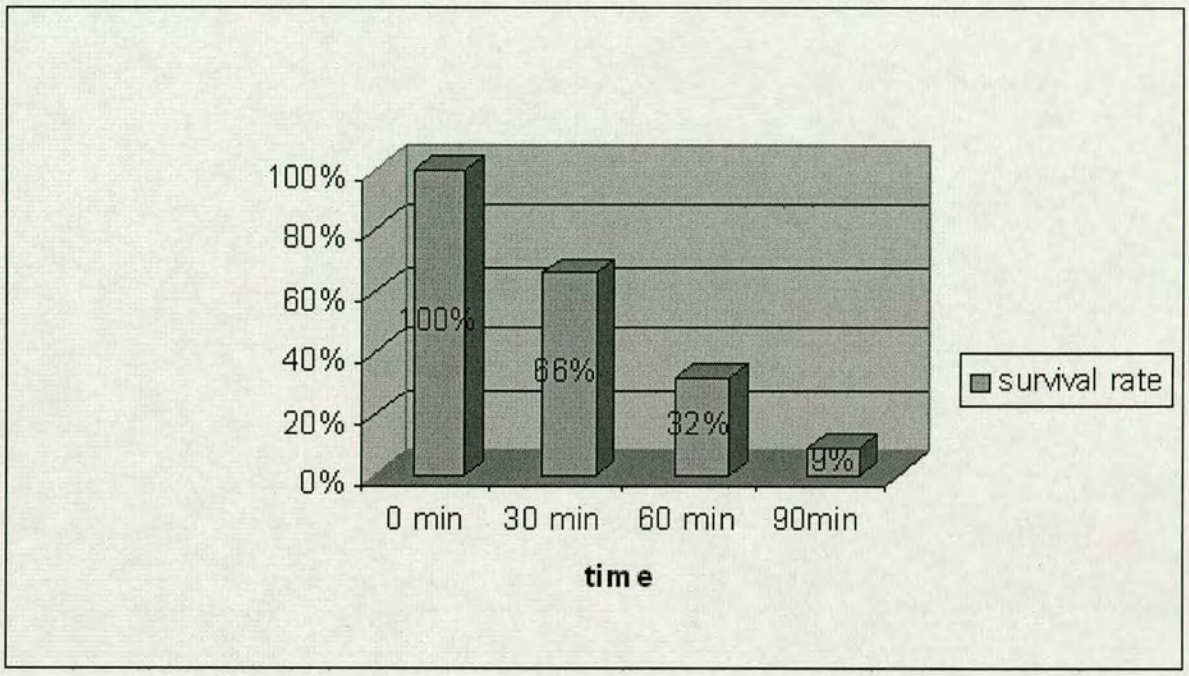


Ed1595 is Ed1538 carring pREP82-cdc37

ED1595 was rescued by the plasmid pREP82-cdc37. Its phenotype looks like WT strain ED0862 at high temperatures on EMMG+leu

ED1538 shows *cdc37* ts phenotype at high temperatures on EMMG+leu+ura

Figure 3.3 Survival rate of ED1595 after MNNG treatment



A final concentration of 1.5 x 10^8 cells/ ml. 700 µl cell suspension was mixed with 300 µl 1 mg/ml MNNG in the same buffer and incubated at 30 ℃ for 30, 60, 90 minutes. The mutagenised cells were resuspended in 1ml EMMG and incubated for 4 hours at 28 ℃ to allow the cells to recover. The samples were spread onto media for counting the survivors after serial dilution.

ED1595 was grown up overnight at 28 ℃ in 200 ml EMMG liquid medium supplemented with leucine. The next day, cells were grown to OD600=0.4, equivalent to a cells density of $5x10^6$ cells /ml. 10 ml cells were harvested by centrifugation at 3000 rpm for 5 minutes and washed once with TM buffer. The pellet was resuspended in TM buffer at a final concentration of 1.5 x 10^8 cells/ ml. 700 µl cell suspension was mixed with 300 µl 1 mg/ml MNNG in the same buffer and incubated at 30 ℃ for 30, 60, 90 minutes with occasional vortexing. 100µl cells was removed and diluted with 900µl TM buffer, then washed twice with 1ml of fresh EMMG medium. The mutagenised cells were resuspended in 1ml EMMG and incubated for 4 hours at 28 ℃ to allow the cells to recover. The samples were spread onto media for counting the survivors after serial dilution.

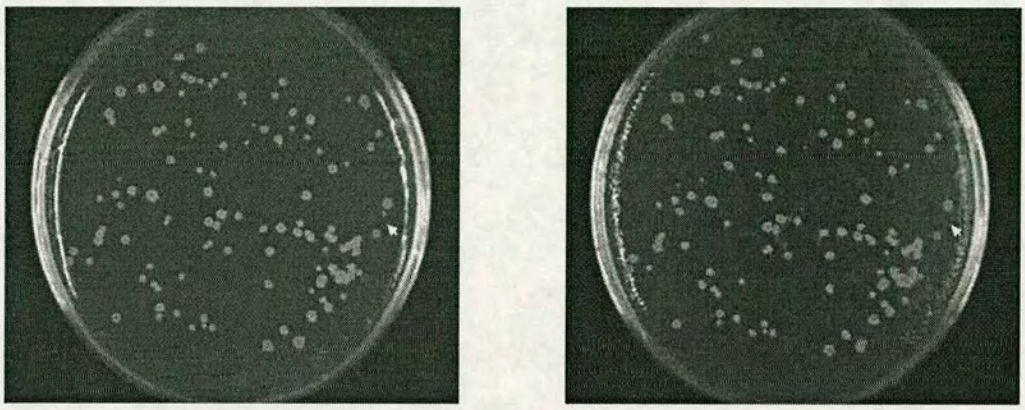
As the data shows 30 minutes treatment and 60 minutes treatment give the kill rate 34% and 68% (Figure 3.3). They were in the middle of the ideal kill rates, which vary from 50%- 90%. So they can be used to screen the synthetic lethal mutants. 90 minutes treatment gives the viable rate as low as 9 %. The mutants obtained using this viable rate may have multiple mutations in several genes and so this batch of cells was discarded. Glycerol was added to aliquots of the 30 and 60 minute batches at a final concentration of 25%. The cells were kept at -70 ℃ for subsequent screening. To estimate how many cells were still alive after freezing, I also plated the cells on medium from the frozen tubes and counted the colonies. The result shows that approximately 1.7 x 10^4 viable cells/ ml were recovered from 30 minutes treatment and 1.1 x 10^4 viable cells/ ml from 60 minutes treatment. The data would be helpful for calculating the volume of cell suspension to plate on medium to produce 200 colonies /plate for screening.

3.5 Screening for synthetic lethal mutants

In theory, synthetic lethal mutants cannot survive either loss of the pREP82-cdc37 plasmid or reducing the expression of the *cdc37* gene on the plasmid. The expected mutant cell population will include strains with an additional mutation either in *cdc37* gene itself or in a different gene that is synthetically lethal with *cdc37*. As described above, both 5-FOA and thiamine can be used as the selective agent. In each case, the synthetic lethal mutants would be scored as either 5-FOA sensitive or thiamine sensitive colonies.

The 68% kill rate cells were plated for screening. An aliquot of the stored cells was diluted to give 200 colonies per plates. Through calculation and undertaking pilot experiment, one aliquot of cells was found to grow on EMMG medium to form 200 viable colonies for total 50 plates. About 10000 colonies can be screened for plating one time. For 5-FOA selection, cells were replicated onto EMMG+leu+ura+5-FOA plates and incubated for 2 days at 28 °C. The replicated 5-FOA plate was compared with the master plate to detect the colonies that did not grow on 5-FOA (Fig 3.4). For thiamine selection, the master plates were made by incubation on EMMG+Leucine for 3 days. They were replicated on to EMMG+leu+Thiamine, grown for one day and then replicated again onto the same medium. This will allow cells enough time to exhaust the Cdc37 protein during growth in the absence of thiamine(Westwood et al., 2004). Through screening more than 40,000 colonies, 68 strains were selected for their 5-FOA or thiamine sensitivities. When retested (Fig 3.5), 15 strains still showed 5-FOA or thiamine sensitivity (Table 3.1).

Figure 3.4 screen for synthetic lethal mutants

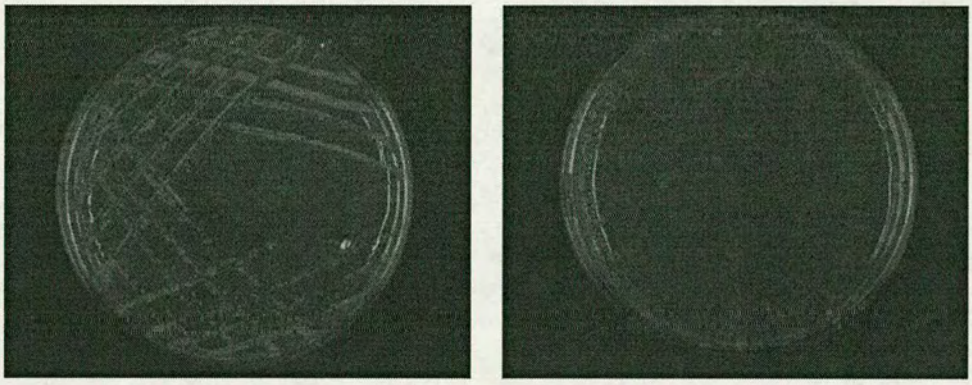


EMMG+leu

EMMG+leu+ura+5-FOA

The arrows point to a colony which grows on non-selective medium, but cannot grow on selective medium. The colony was picked for retesting.

Figure 3.5 the selected mutant was retested for 5FOA sensitivity



On EMMG+ura

On EMMG+ura+leu+5FOA

The synthetic lethal mutants show 5-FOA sensitivity and grow well on EMMG medium without 5-FOA

Table 3.1 Summary of selected mutants

Selective agent	First selection	Retest		
		no growth on selective medium	grow on selective medium	no growth on non-selective medium
5-FOA	35	10*	17	8
Thiamine	33	5*	24	4

As can been seen from the table above, more 5-FOA sensitive strains were identified than thiamine sensitive strains. Both 5-FOA and thiamine sensitive strains were tested for sensitivity on both selective agents.

*strains indicated with an asterisk were retained for further study

Table 3.2 Summary table of mutants showing sensitivity on both selective agents

Isolate	Strain name	Selective agent	5-FOA	Thiamine
ED1595			R	R
F08	J102	5-FOA	S	S
F15	J105	5-FOA	S	S
F17	J106	5-FOA	S	S
F23	J204	5-FOA	S	S
F29	J216	5-FOA	S	S
F31	J217	5-FOA	S	S
F34	J218	5-FOA	S	S
F41	J222	5-FOA	S	S
F47	J231	5-FOA	S	R
F52	J242	5-FOA	S	R
T15	J304	Thiamine	S	R
T27	J306	Thiamine	S	S
T34	J322	Thiamine	S	S
T43	J403	Thiamine	S	S
T55	J405	Thiamine	S	S

S = sensitive

R= resistant

This demonstrated that the experimental methods were able to generate mutants of the desired type. A total of 15 mutants were tested for both phenotypes. 12 of 15 mutants had 5-FOA^S and Thiamine sensitive. It indicated that the two different approaches worked well for screening (Table 3.2). The promising mutants which had both sensitivity phenotypes were frozen down. Using 5-FOA as the selective agent seems more efficient than thiamine because 10 of 15 mutants were isolated by 5-FOA. One possible explanation is that mutants need time to use up the Cdc37 protein in *vivo*, so it takes more time for cells to present thiamine sensitivity. Furthermore, the plates had to be replicated twice, which resulted on colonies being spread out on the second replica, causing difficulty in identifying sensitive colonies. So the subsequent analysis only used 5-FOA.

3.6 Analysis of mutants

ura4 plasmid results in low frequency integration in the chromosome after transformation (Forsburg et al, 2001). The resulting strain would also exhibit 5-FOA sensitivity, not because of mutations which are synthetic lethal with *cdc37* and cause cell death, but because 5-FOA kills the cells which carry the integrated *ura4*$^+$ gene. To identify the strains that had the integrated plasmid, a plasmid shuffle experiment was carried out (Fig 3.6). The synthetic lethal mutants were maintained by a wild type copy of the gene on the pREP82-cdc37 plasmid carrying the *ura4*$^+$ gene complementing the uracil auxotrophy of the strain. After transformation of pREP81-cdc37 constructs (leu$^+$) or pREP1 as control (leu$^+$) into synthetic lethal strains (carrying pREP82-cdc37), cells carrying both plasmids were plated out on 5-FOA medium. Strains which become 5-FOA resistant with pREP81-cdc37 and 5-FOA sensitive with pREP1 were identified as synthetic lethal strains. As described above, plasmid integrated strains

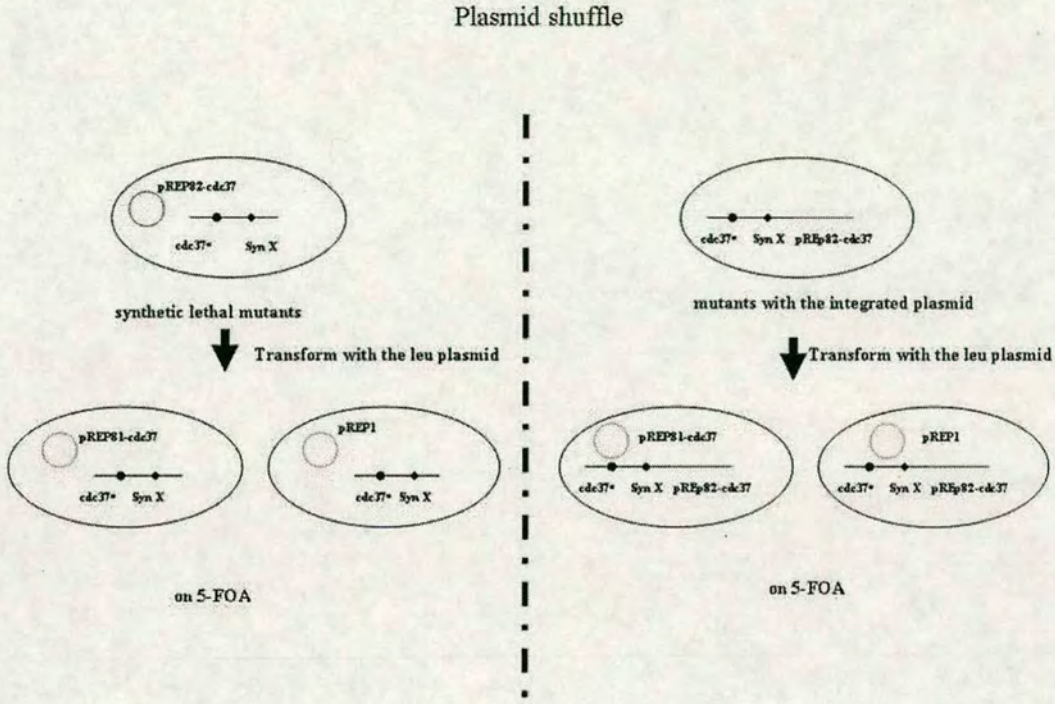
Table 3.3 Summary table of mutants that were tested by plasmid shuffle

Mutants	pRep81-cdc37 (a)	pRep1 (a)
J102	S	S
J05	R	S
J06	R	S
J204	R	S
J216	R	S
J217	S	S
J218	R	S
J222	R	S
J306	R	S
J322	R	S
J403	R	S
J405	R	S

All mutants were tested on EMMG+Leu+5FOA at 28 ºC

R= resistant S= Sensitive

Figure 3.6 synthetic lethal mutants were tested by plasmid shuffle



Mutants were kept alive by transforming ura based plasmid carrying cdc37^{+}. Mutants were transformed with leu based plasmid (pREP81-cdc37). The transformants *cdc37-681 synX 1eu 1ura4* [pREP82-*cdc37*] [pREP81-*cdc37*] can lose the pREP82-*cdc37* plasmid (it has pREP81-*cdc37*) and become ura4^{-}, 5-FOA resistant. The transformants *cdc37-681 synX 1eu 1ura4* [pREP82-*cdc37*] [pREP1] cannot lose the pREP82-*cdc37* plasmid and become ura4^{+}, 5-FOA sensitive. Plasmid integrated mutants cannot be rescued by pREP81-*cdc37* and must remain ura4^{+}, 5-FOA sensitive.

were killed by 5-FOA because they carry the *ura4*$^{+}$ gene. This cannot be rescued by the wild type version of *cdc37*, expressed from pREP81-cdc37. So, strains showing 5-FOA sensitivity with both pREP81-cdc37 and pREP1 are probably plasmid integrated strains. Total 12 mutants were tested, Mutants J102 and J217 were 5-FOA^{S} with pREP81-cdc37 and pREP1 suggesting the plasmid had integrated into the chromosome.

3.7 Characterization of synthetic lethal strains

The temperature *cdc37* sensitive phenotype of the mutants can be complemented by the plasmid carrying a wild copy of *cdc37* gene. Any additional phenotypes, such as temperature conditional growth, should be attributed to the synthetic lethal mutations. It was possible that these mutants would exhibit a conditional growth defect.

Therefore, 10 mutants were examined for cold sensitive or temperature sensitive growth phenotype at 20 ℃or 36 ℃. Most of the mutants do not exhibit cold sensitive or temperature sensitive phenotype. J306 and J322 seem specially interesting. At high temperature J306 cells appeared rather round and fat and J322 cells were slightly elongated. It indicated that the two mutants had phenotypes of their own, although they were not obvious or strong.

3.8 Outcross of the mutants

It is possible to cross the synthetic lethal mutations directly out of the mutants by tetrad dissection. Each mutation can be checked for whether it had a phenotype of its own. If different synthetic lethal mutants showed the same phenotype, they may be mutated in the same gene. It will make further investigation easier if the mutations have phenotypes of their own.

The cross can be carried out between each synthetic lethal mutant with wild type strain ED0862. After dissection and plating on YE plates, most of the progeny should have 3 viable spores. Then the spores are plated onto EMMG+Leu to test for Ura^+. None of the 3 viable spores should be ura^+. Then they are incubated at 36 °C and examined under microscope. One of them should look like *cdc37* ^{ts} phenotype, one look like wild type and the other one should look like the phenotype of its own. If 4 viable spores in one tetrad appear after dissection, one of the spores should be Ura^+, because the spore carries the plasmid.

Table3. 8 The expected genotype and phenotype of the progeny in tetrads from *J322* x *ED0862*

1) Assuming all spores have lost the plasmid

Type of Tetrad	Genotype of the progeny	Phenotype of the progeny
Parental ditype	2 *cdc37syn322* 2 wildtype	2 dead spores 2 wildtype spores
Tetratype	1 wildtype 1 *cdc37* 1 *syn322* 1 *syn322 cdc37*	1 wildtype 1 *cdc37* ? 1 dead
Non-parental ditype	2 *cdc37* 2 *syn322*	2 cdc37 ?

2)

1) Assuming some spores have the plasmid

Type of Tetrad	Genotype of the progeny	Phenotype of the progeny
Parental ditype	2 *cdc37syn322* 2 wildtype	1 or 2 wild type like spores 2 wildtype spores
Tetratype	1 wildtype 1 *cdc37* 1 *syn322* 1 *syn322 cdc37*	1 wildtype 1 cdc37 or 1 wild type spores ? 1 wild type like spores
Non-parental ditype	2 *cdc37* 2 *syn322*	1 or 2 wild type spores with 1 cdc37 ?

Table 3.9 phenotype of synthetic lethal mutant in cdc37^{+} background

Strains	28 ℃ (a)	36 ℃ (a)	Backcross with cdc37 ts
J05	NORMAL	NORMAL	YS
J06	NORMAL	NORMAL	YS
J18	NORMAL	NORMAL	YS
J202	NORMAL	NORMAL	YS
J204	NORMAL	1	YS
J210	NORMAL	2	YS
J216	NORMAL	NORMAL	YS
J306	Did not cross	N/A	N/A
J322	NORMAL	3	YS
J405	Did not cross	N/A	N/A

a) on YE plates

1= a few cells in the colony are a bit lysis and slightly elongation

2= a few cells in the colony are a bit lysis and slightly elongation

3= most cells are elongation and totally lysis

YS= cross successfully and the additional mutations in mutants are synthetic lethal with *cdc37-681*

10 strains were backcrossed with WT to separate synthetic lethal mutation out of cdc37 ts background. From the data show 8 strains were cross well. J306 and J405 did not cross. These may be caused by the additional mutations in the mutant that are required for mating or meiosis. They may result in the defect to produce the spores. The mutations in J204 and J210 induce similar phenotypes. That is, a few cells in the colony show some lysis and slight elongation at high temperature. Whether they have the same mutation needs to be test. J322 is interesting because the mutation from J322 has a strong phenotype at 36 ℃ .

4. Identification of candidate genes

4.1 General introduction

In the previous chapter, experiments were done to isolate synthetic lethal mutants and to understand the nature of the isolated mutants by genetic methods. The temperature sensitive strain *cdc37-681* was used as the starting strain. It was transformed with a plasmid pRep82 expressing the wild type version of *cdc37* and mutagenised. Then selective agent 5FOA was used to screen for strains which rely on the plasmid for survival. A total of 12 mutants were isolated among 40,000 colonies. Two of the 12 strains were identified as plasmid integrated mutants. In the final, 10 synthetic lethal mutants were analysed by outcrossing to investigate the properties of the additional mutation in the mutants.

Little information was obtained by these methods to understand the character of the mutation in the mutants.

4.1.1 Strategy

To clone the wild type version of the gene that is mutated in the synthetic lethal strain, a plasmid-based screen is the most common technique. In this project, plasmid shuffle system is applied to identification of the gene which is defective in the

mutants. To carry out this in practice, the synthetic lethal mutants are transformed with a library of plasmids with *leu*$^+$ selective marker. When Leu$^+$ plasmid was transformed in the mutants, the original ura$^+$ plasmid carrying wild type *cdc37* gene will be shuffled out of the transformed mutants. If the transformed cells are still alive on 5FOA media, it indicates that the gene from *leu*$^+$ plasmid can rescue the defect of the chromosomal *synX* mutation.

As described in the previous chapter, mutants were screened for two possible situations. One is that the additional mutation *synX* combining with *cdc37*ts causes cell death at permissive temperature, kept alive by the expression of Cdc37 protein from plasmid with *ura4*$^+$ marker. The other is that the mutation occurs in *cdc37* itself and abolishes its function, in which case the cells also need the plasmid for survival.

It is worth mentioning that *cdc37* gene itself in the leu plasmid library would also rescue synthetic lethal mutants. However *cdc37* gene may be cut into truncated versions during the making at the genomic libraries were made because it contains *Hin*dIII, *Bam*HI and *Spe*I sites within the gene. And the truncated versions of *cdc37* are unlikely to rescue the mutants.

4.1.2 Vector and genomic libraries

The vector pAL-KS has been used for constructing genomic libraries in *S. pombe* (Terasawa et al., 2006). It has the resistant marker Amp^r which is used to select the plasmid in *E.coli*. The vector also contains two elements from yeast, the nutritional selectable marker *LEU2* from *S. cerevisiae* and a *S. pombe* origin sequence for plasmid replication in yeast. Multiple cloning sites are located between two *Pvu*II sites for the insertion of DNA fragments.

In this project, genomic libraries were selected to use rather than cDNA libraries due to the different properties of the two kinds of library. Genomic libraries allow modest overexpression; a potential candidate gene in genomic library which can rescue the mutant may express properly to fill the need of the cell. The genes in the most commonly used *S. pombe* cDNA libraries are expressed much more strongly than normal level *in vivo* (Jones et al., 1988). It increases the chance of getting false positive clones during the screening. To get a good representation of genes from cDNA library, many more clones need to be screened.

4.2 Transformation of genomic libraries into synthetic lethal mutants

The synthetic lethal mutants were maintained on EMMG+leucine by a wild type copy of *cdc37* gene on the plasmid carrying the *ura4*$^+$ gene complementing the uracil auxotrophy of the mutant. When the mutants are transformed with a genomic library of plasmids containing *leu*$^+$ selective marker, transformants will grow on EMMG+uracil medium. After replication on EMMG+uracil+5FOA, most of the transformants cannot survive, because 5FOA selects against original plasmid pREP82-Cdc37 containg *ura4*$^+$ gene. Loss of this plasmid results in the mutants failing to survive without the Cdc37 protein. A small proportion of cells can grow on EMMG+uracil+5FOA if the gene carried by leucine genomic library plasmid can compensate for the loss function of the *syn*X mutation (Figure 4.1).

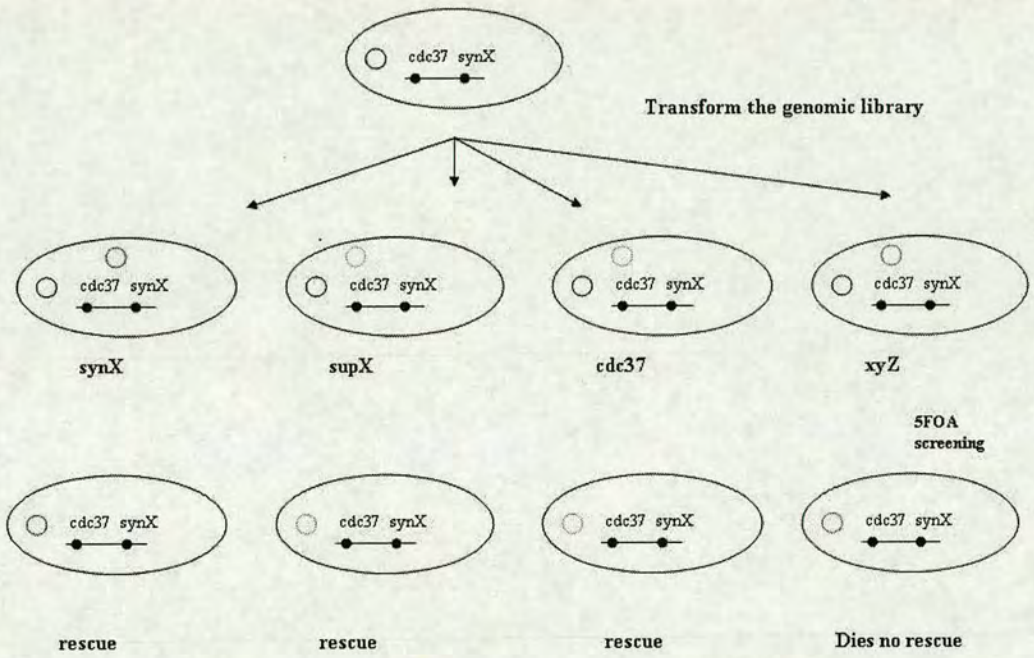
Pre-culture was then inoculated into 100 ml EMMG+Leu overnight at 28 ℃ in the shaking incubator. The OD_{600} of the culture was checked to be sure that it was

about 0.4 before transformation. For each transformation, 1 μl of a 2.0 μg/ μl stock of the *Bam*HI library and 1.5 μl of 1.7 μg/μl stock of the *Hin*dIII library, 1.5 μl of the 1.6 μg/μl *Spe*I library were used to transform the mutants. A no-DNA control was also included. After electroporation, 200 μl aliquots of each diluted sample were then plated onto 4 EMMG+uracil plates. The plates were incubated at 28 ℃ for four days to allow colonies to form. There were around 3000-4000 colonies per plate, which was enough to cover the whole genome during one transformation.

Each plate was then replicated onto EMMG+Ura+5FOA to select the colonies in which the *leu* library plasmid carries a gene able to rescue the mutant. Such colonies can lose the *ura* plasmid carrying wild type *cdc37*. Under most circumstances, there were only a few colonies (usually less than 10 per plate) able to survive on EMMG+Ura+5FOA. Once such 5FOA colonies appeared, they were picked to test on EMMG+Ura+5FOA again to ensure their resistance.

The resistant clones were incubated at 28 ℃ in 10 ml EMMG+Ura media. Plasmids in these clones were recovered from the yeast cells to transform into *E. coli,* because it is easier to isolate the plasmids in bacteria than yeast for further studies.

Figure 4.1 The synthetic lethal mutants were transformed with plasmid library Color codes for plasmids.



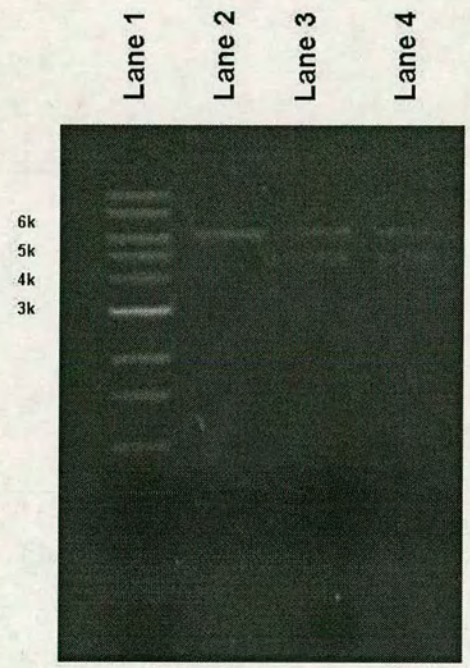
The synthetic lethal mutants were kept alive by uracil plasmid carrying *cdc37* gene. After transformation and selection on 5FOA, cells which had lost the uracil plasmid and contain the leucine plasmid were identified. A) The *synX* or suppressor gene was present in the leu genomic library and can compensate the loss function of the syn mutation, cells were alive and present 5FOA resistance; B) *cdc37* gene in leu genomic library also can compensate the loss function of the *cdc37* mutation, cells were alive and present 5FOA resistance. C) irrelevant gene xyZ in leu genomic library can not rescue the mutant, cells were dead and present 5FOA sensitive.

4.3 Investigation of candidate gene in J05 and J06

The transformed J06 cells showing plasmid-conferred resistance to 5FOA were identified according to the procedure explained above. A total of eight strains with plasmid-conferred resistance were identified in 5FOA screen. Of these, five were transformants from the *Hin*dIII library and three from the *Bam*HI library. Two plasmids from *Hin*dIII library could rescue the J06 mutant, but the others could not (data not shown). Restriction enzyme analysis was carried out on the rescuing plasmids (Figure 4.2).

After transformation of strain J05, a total of eleven strains with plasmid-conferred resistance were identified in 5FOA screen. Of these, six were from *Hin*dIII library and five from the *Bam*HI library. All samples were transformed back into the mutant to test whether they could rescue. The result show only plasmid J05-H3 and J05-H4 from *Hin*dIII library could rescue the strains. The restriction enzyme analysis was carried out on the samples (Figure 4.3). The inserts from plasmids rescuing J05 and J06 appear very similar in size.

Figure 4.2 Restriction enzyme analysis of plasmids recovered from J06 mutant



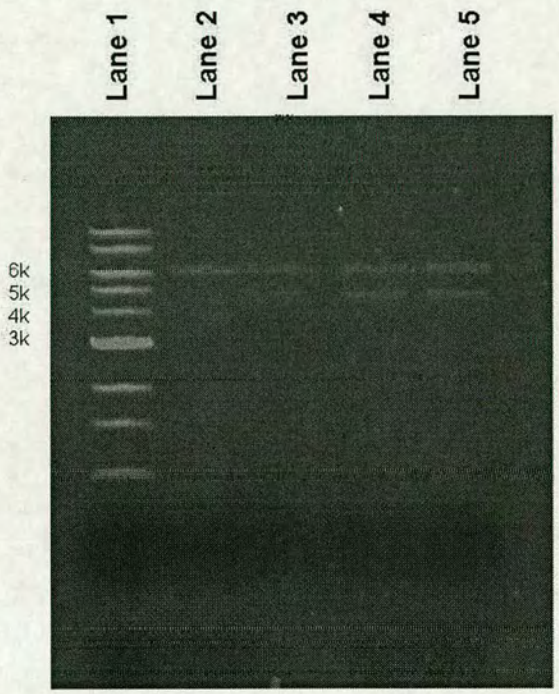
Lane1: 1kb ladder DNA Marker

Lane2: empty vector digested by *Hin*dIII

Lane2: J06-H2 digested by *Hin*dIII

Lane3: J06-H3 was digested by *Hin*dIII

Figure 4.3 Restriction enzyme analysis of plasmids recovered from J05 and J06 mutants



Lane1: 1kb ladder DNA maker

Lane2: empty vector was digested by *Hin*dIII

Lane3: J05-H3 was digested by *Hin*dIII

Lane4: J05-H4 was digested by *Hin*dIII

Lane5: J06-H2 was digested by *Hin*dIII

The genomic fragments in plasmids may contain genes or gene fragments that can rescue the mutants. Sequencing the genomic insert is the best way to investigate which gene is involved. The sequencing was done using the primer M13-21 forward and reverse universal sequencing primers (Sigma). The sequencing reactions were run by University of Edinburgh sequencing service center. The original sequence data retrieved from each primer was examined using Bio Edit software (Tom Hall Company).

Sequence of the plasmids J06-H2 by using the forward primer was done by BLAST search at NCBI, the first 100 base pairs of the sequence show high similarity with the vector sequence and the latter 500 bp high similarity with the *S. pombe* genes (Figure 4.4 and Figure 4.5). A similar result comes from the sequence using the reverse primer. It indicates that the sequence between both ends of the vector sequence is the genomic DNA insert.

The vector sequence was identified and removed so that the "clean" insert sequence was retained for further investigation. The "clean" insert sequences were run through a DNA BLAST search at NCBI database (The National Center for Biotechnology Information) and *S. pombe* database at the Sanger Centre website (http://www.sanger.ac.uk). It allows identification of the genomic region that each insert spanned. Further information on each of the genome fragments was accessed through the Gene Database at website (http://www.genedb.org/genedb). The results show that both primers gave sequence from the same chromosomal region, with distance and orientation consistent with the restriction analysis.

Figure 4.4 Alignment of J06-H2 sequence obtained with chromosomal sequence in *S. pombe* database

```
Query:       1 CGAACAACGCACATTGACGACTTTAAACAGAATTATTCATCAAGCTAAGCAAACAATTTC 60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1940638 CGAACAACGCACATTGACGACTTTAAACAGAATTATTCATCAAGCTAAGCAAACAATTTC 1940697

Query:      61 CGAAAATGCTCAAGCTTTTGAGGAAATGAAACTTCCTACCTACGAAGATAAATTGCTACC 120
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1940698 CGAAAATGCTCAAGCTTTTGAGGAAATGAAACTTCCTACCTACGAAGATAAATTGCTACC 1940757

Query:     121 TCTCGTGCGTTTTCCTATTAAGTTGTTGGAAGAGGCGCTTCGTCTTCGCCTGGCATACGC 180
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1940758 TCTCGTGCGTTTTCCTATTAAGTTGTTGGAAGAGGCGCTTCGTCTTCGCCTGGCATACGC 1940817

Query:     181 CAAGAAAATTAAAGGACCTAATTTTTTAATTGTAGATTCTATGTTAGATGATTTCAAAAT 240
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1940818 CAAGAAAATTAAAGGACCTAATTTTTTAATTGTAGATTCTATGTTAGATGATTTCAAAAT 1940877

Query:     241 CGCGTTGTCTGTGGCAGTACGCATAAAGCGCGAATACATAAAAATTGCGAGCCCATCACC 300
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1940878 CGCGTTGTCTGTGGCAGTACGCATAAAGCGCGAATACATAAAAATTGCGAGCCCATCACC 1940937

Query:     301 GGGTTGGAGTTTACCAACAAATGTGGATGAAGATTATGATAACGTGTTACTTGACTCCTT 360
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1940938 GGGTTGGAGTTTACCAACAAATGTGGATGAAGATTATGATAACGTGTTACTTGACTCCTT 1940997

Query:     361 GAAATTTTACTTTAAACTTCTTACTTTGAAACTAAGTTCAGGGAACAAGAATCTATATTT 420
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1940998 GAAATTTTACTTTAAACTTCTTACTTTGAAACTAAGTTCAGGGAACAAGAATCTATATTT 1941057

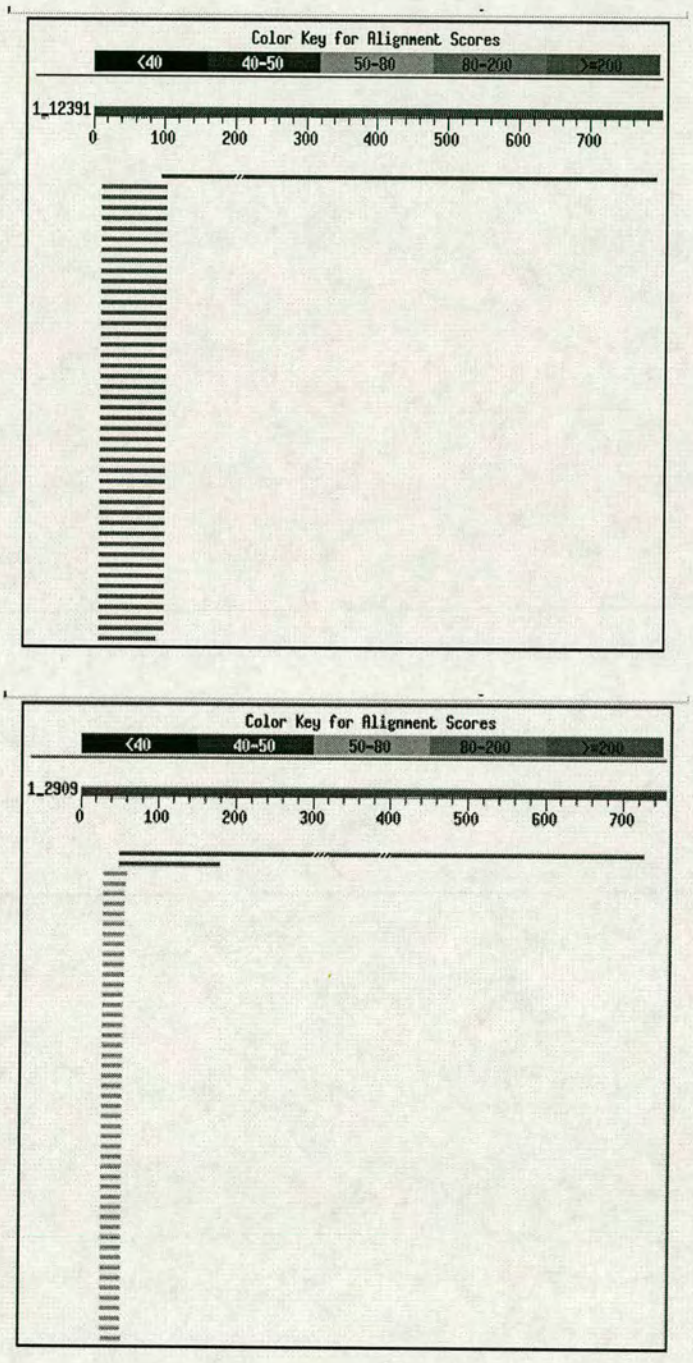
Query:     421 TAAGGAAATCGACTTTTTGGAAAATGAGTGGGCATTTTTAAATGAACACATCTATTGGAT 480
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1941058 TAAGGAAATCGACTTTTTGGAAAATGAGTGGGCATTTTTAAATGAACACATCTATTGGAT 1941117

Query:     481 CAATGGTGGTGATATTCATATGGCTGGACAATTTAGTTACCTATCAAATAGTCTGTTGCT 540
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1941118 CAATGGTGGTGATATTCATATGGCTGGACAATTTAGTTACCTATCAAATAGTCTGTTGCT 1941177

Query:     541 TAATGTTCATCGTTATGTGGAGTCGCATCTCAATGGACCAACAGAACGTACTGCCG 596
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1941178 TAATGTTCATCGTTATGTGGAGTCGCATCTCAATGGACCAACAGAACGTACTGCCG 1941233
```

The top line is the sequence of the J06-H2 by forward primer. The bottom line is the sequence of the chromosome in the database.

Figure 4.5 BLAST alignment of sequences derived from J06-H2 using M13-21 forward and reserve primers



The result shows that the sequences have similarity with the chromosomal seqeunce

in the database. The short line represents the part of the vector sequence, the long line represents the gene

The next step in the analysis was to identify genes located within the cloned insert sequence. To do this, for each plasmid insert, sequences derived from the forward and reserve primers were used to BLAST search the *S. pombe* database. The results will show which part of the sequence in the yeast chromosome corresponds to the sequence of the isolated plasmid. From the genomic region which was spanned by each insert, we can identify which genes or genes are likely to be involved.

The plasmids J06-H2 and J06-H3 were isolated from the synthetic lethal mutant J06. In DNA gel, it can be seen that the insert of the two plasmids have the same size (Figure 4.2). The two sequences of the inserts were the same. When the sequence was used in a BLAST search with the *S. pombe* database, I found that the genomic region which was spanned by the insert was located in chromosome 1 with contig c977 and contig location between 1940638 and 1944936 (Figure 4.6). The truncated *wis4* encoding a MAP kinase kinase kinase (MAPKKK) and a small gene rpl3001 encoding 60S ribosomal protein L30 locate in the region (figure 4.6).

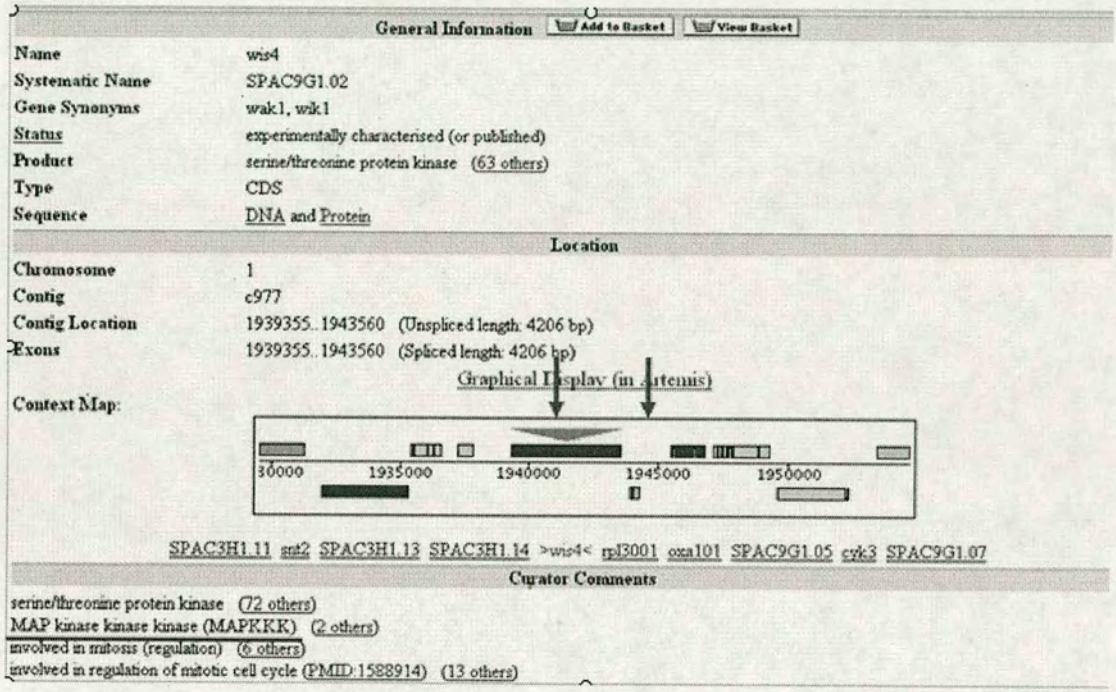
The plasmid J05-H3 and J05-H4 were isolated from the synthetic lethal mutant J05. Retransformation of the plasmid shows that both of the plasmids can rescue original mutant J05. In DNA gel, it can be seen that the insert of the two plasmids have the same size (Figure 4.3). The two sequences of the insert of plasmids were the same. The genomic region which was spanned by the insert was located in chromosome 1 with config c977 and contig location is between 1940638 and 1944936. The truncated *wis4* encoding MAP kinase kinase kinase (MAPKKK) and a small gene rpl3001 encoding 60S ribosomal protein L30 locate in the region (figure 4.6).

After comparing with the sequence from J05-H3 and the sequence that I got from J06-H2, I found that the exactly the same DNA fragment was isolated twice from different strains. The plasmids which carry truncated *wis4* can suppress the J05 and J06 mutants. It suggests that *wis4* may be potential candidate gene. It seems that the insert contains a N-terminal truncated version of the *wis4* gene. *wis4* encodes a MAP kinase kinase kinase (MAPKKK). It belongs to the serine/threonine protein kinase family, and is involved in a signal transduction pathway that is activated under various stress conditions (Samejima et al., 1997).

I wondered whether the intact version of the gene was able to rescue the mutant. Plasmid shuffle experiment was applied to test it. A series of *leu*$^+$ plasmids which carry the authentic gene were transformed into the mutant. The aim of the experiment was to detect whether the rescued plasmids in the mutant can be shuffled out by the plasmids carrying the authentic gene.

Fortunately, plasmids expressing *wis4* had been constructed in this laboratory (Samejima et al., 1997). The plasmids pWF96 and pWF48 which were constructed in the pREP1 vector were used to test the J06 mutant. pWF96 contains the full length of *wis4* and pWF48 encodes the kinase catalytic domain of Wis4. After transformation, it was found that both pWF96 and pWF48 can rescue J06 as well as J06-H3 can do. pWF48 can suppress the mutant better than pWF96 (Figure 4.7).

Figure 4.6 Location of the J06-H2 insert sequence in *S. pombe* genome



The BLAST result shows which genes are present in the isolated fragment.. The two blue arrows indicate the position of the ends of the J06-H2 insert

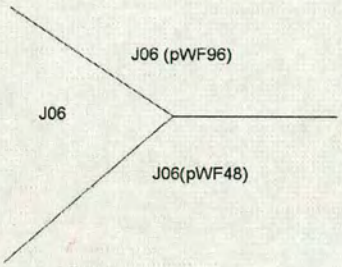
Through the functional analysis of *wis4* gene, it is known that the N-terminal of gene product interacts with negative regulator Mcs4 and affects Wis4 kinase activity (Cottarel, 1997). The insert that I isolated from the mutant is an N-terminal truncation. It is possible that the Wis4 kinase activity will be higher if the N-terminal domain is absent. The truncated version of *wis4* may suppress the mutant better and be identified more easily during the screen.

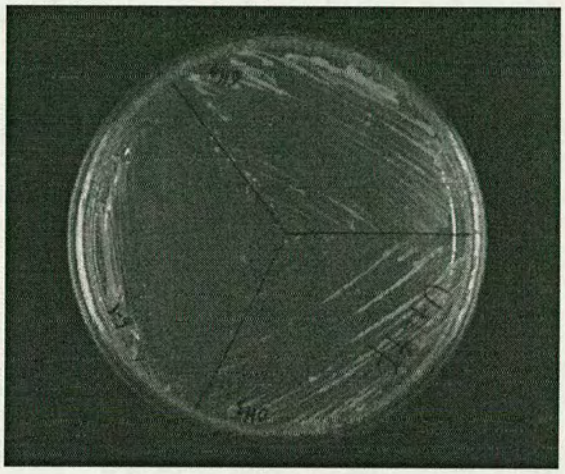
From the previous experiment, we know that *wis4* is able to rescue the mutants J05 and J06. I wondered whether the mutated gene in the synthetic lethal strains is this gene. Genetic analysis was applied to test whether *wis4* is synthetically lethal with *cdc37ts* by crossing a *wis4* deletion strain with *cdc37ts* strain. Spore colonies which have the defects from both parent strains will be dead if there is a synthetic lethal interaction. In some cases, the spores which have both defects in one of isolated gene and c*dc37-681* were alive. This indicates that the *wis4* deletion strain does not show synthetic lethality with *cdc37-681*.

wis4 encodes a MAP kinase kinase kinase (MAPKKK) and is involved in a signal transduction pathway that is activated in under various stress conditions in *S. pombe* (Samejima et al., 1997). This stress response pathway is composed of three consecutive kinases (MAPKKK, MAPKK, and MAPK). MAPKKK phosphorylates and activates MAPKK, which in turn phosphorylates and activates MAPK (Figure 4.8). Wis4 and Win1 act as the upstream regulators of Wis1 MAPPK (Buck et al., 2001). Wis1 is required for activation downstream of the MAPK homologue Spc1, and the Wis1–Spc1 pathway is required for survival in stressful conditions (Samejima

Figure 4.7 Transformation of wis4 into the mutant

Wis4

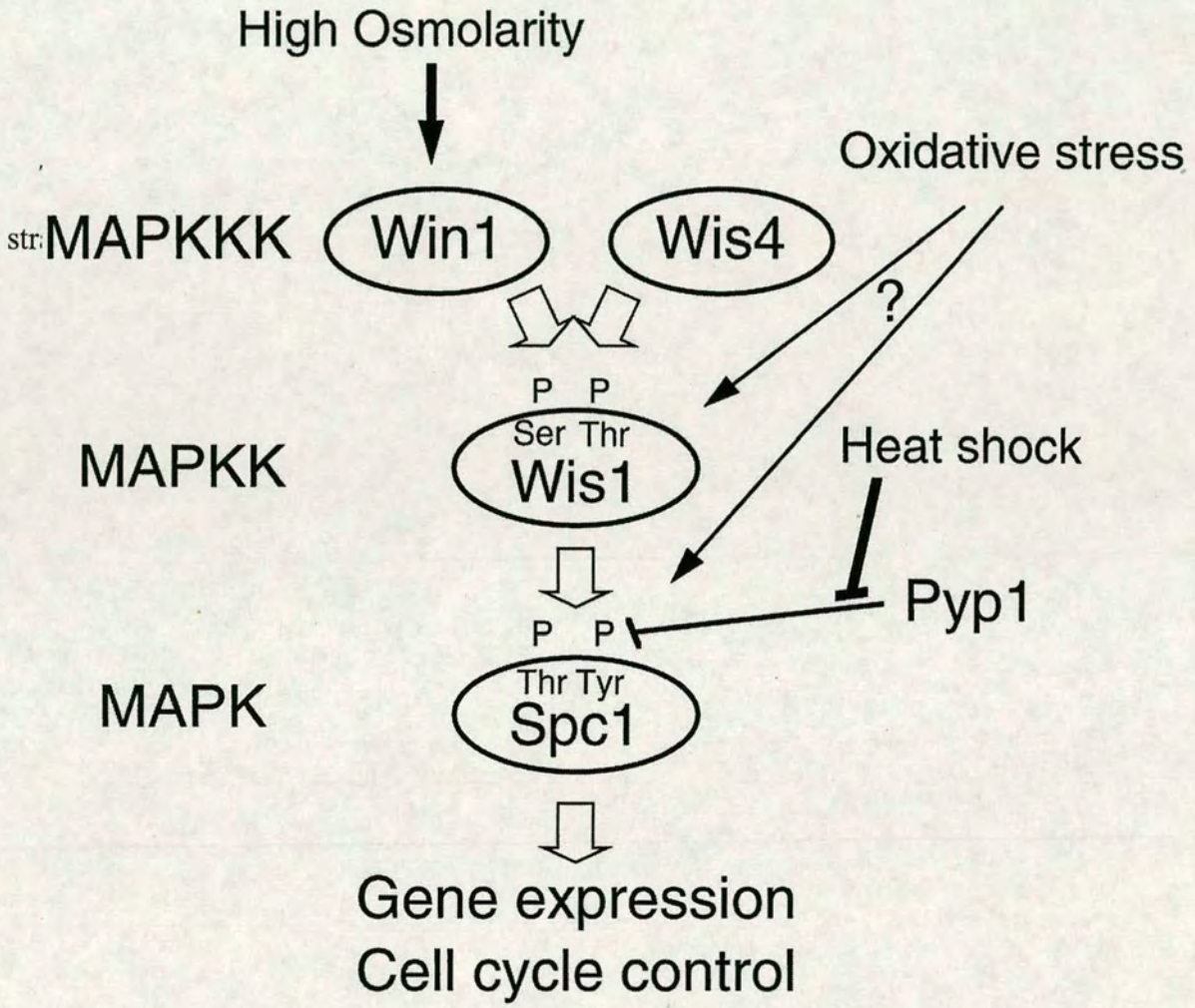




EMMG+ura+5FOA

The transformed cells were streaked on EMMG+ura+5FOA and incubated at 28 °C for 2 days

Figure 4.8 Stress response pathway in fission yeast.



Wis4 MAPKKK phosphorylates and activates Wis1 MAPKK. Wis1 phosphorylates and activates Spc1 which regulates cell cycle and gene expression (Samejima et al., 1998)

et al., 1997).ED1181 in which *wis4* gene was deleted and replaced by *ura4* gene (*wis4::ura4*$^{+}$ *leu1-32*) was crossed with *cdc37ts* strain ED1538 (*cdc37-681 leu1-32 ura4-D18*). Crosses were incubated in Helicase to release the spores (chapter 2). Spores were plated on YE plates and incubated at 28 °C for 4 days. 80 spores were picked and incubated on YE overnight and then replicated onto EMMG+leucine 28°C and YE plates at 36 °C. They were scored for ura^{+} and examined under microscope for *cdc37ts* phenotype (Table4.1).

From the table, it was found that spores present the four groups of phenotype. Specifically, 17 of 80 spores have genotype 4 (*ura*$^{+}$ *cdc37*^{ts}) and it means that the cells which have *wis4* deletion and *cdc37ts* in the chromosome were alive. This indicates that *wis4* deletion and *cdc37-681* are not synthetic lethal.

In some cases, it is perhaps that the cloned genes are the suppressors or the element involved in the same pathway downstream of the mutated gene. The isolated genes may still have the ability to rescue the corresponding defective gene in mutants. But the chromosomal mutation is still unknown.

Wis4 and Win1 act as the upstream regulators of Wis1 MAPKK, acting in parallel (Samejima et al., 1998).I wondered whether other elements such as *win1* gene in MAP kinase pathway were synthetically lethal with *cdc37-681*. The *win1* deletion strain ED1291 in which *win1* gene was deleted and replaced by *LEU2* gene (*win1::LEU2 ura4-D18*) was crossed with *cdc37ts* strain ED1538 (*cdc37-681 leu1-32 ura4-D18*). Crosses were incubated in Helicase to release the spores. Spores were

Table 4.1 Cross of *wis4:: ura4 leu1-32* cross with *cdc37-681 leu1-32 ura4-D18*$^{-}$ by random spores

***ura*$^{+}$*/ura*$^{-}$**

1	2	3	4		5	6	7	8
-	-	-	-		-	-	-	-
-	-	-	-		-	-	-	-
-	-	-	-		-	-	-	-
+	-	+	+		-	-	-	-
+	+	+	+		-	+	-	-
+	+	+	-		+	-	+	+
-	+	+	+		+	-	-	-
-	-	-	+		+	-	-	-
-	-	-	-		+	+	+	-
-	-	-	-		-	-	-	+

- = *ura+* = *ura*$^{+}$

cdc37^{+} */cdc37*^{ts}

+	+	+	-		+	+	+	+
+	+	+	+		+	+	+	+
+	+	+	-		+	+	+	+
-	+	-	-		+	+	+	+
-	-	-	-		-	-	-	-
+	+	-	+		-	-	-	-
-	+	-	-		-	-	-	-
+	+	+	+		+	+	+	-
-	+	+	+		+	+	-	-
-	+	+	+		+	+	+	+

Genotype of the spores

1	1	1	2		1	1	1	1
1	1	1	1		1	1	1	1
1	1	1	2		1	1	1	1
4	1	4	4		1	1	1	1
4	4	4	4		2	4	2	2
3	3	4	1		4	2	4	4
2	3	4	4		4	2	2	2
1	1	1	4		3	1	1	2
2	1	1	1		3	3	4	2
2	1	1	1		1	1	1	3

1=*ura*$^{-}$ *cdc37*$^{+}$ 2=*ura*$^{-}$*cdc37*^{ts} 3=*ura*$^{+}$*cdc37*$^{+}$ 4=*ura*$^{+}$*cdc37*^{ts}

plated on YE plates and incubated at 28 °C for 4 days. The table shows the phenotypes and deduced genotype of 80 random spores from the cross. 80 spores were picked, incubated on YE and then replicated onto EMMG+uracil 28°C and YE plates at 36 °C.

They were counted for Leu^+ and examined under microscope for *cdc37ts* phenotype (Table 4.2). The results show 14 of 80 spores appear genotype 4 (leu^+ $cdc37^{ts}$) and it means that the cells which have *win1* defect in combination with *cdc37ts* were alive. It suggests that the synthetic mutation in J05 and J06 is not in *win1* gene either.

Table4.2 Cross of *win1::LEU2 ura4-D18* x *cdc37-681 leu1-32 ura4-D18* - by random spores

leu$^+$/leu

1	2	3	4		5	6	7	8
-	+	-	+		+	-	+	+
-	+	-	+		+	-	+	+
-	-	+	-		+	+	-	+
-	+	+	+		-	+	+	+
+	+	+	-		+	+	+	-
+	-	+	+		+	+	+	+
+	+	+	+		-	+	-	-
-	-	-	-		-	+	+	+
+	+	-	+		-	+	-	+
-	-	-	+		-	-	+	+

- = *leu+* = *leu$^+$*

-	-	+	+		+	+	+	+
+	+	+	-		+	+	+	+
+	+	+	+		-	+	+	+
+	-	+	+		+	-	+	-
+	+	+	+		-	+	-	+
-	-	+	+		+	+	+	+
-	+	+	-		+	-	+	+
+	+	+	+		-	+	-	+
+	-	+	+		+	-	+	+
+	+	+	-		+	-	+	+

cdc37$^+$ /cdc37^{ts}

Genotype of the spores

2	4	1	3		3	1	3	3
1	3	1	4		3	1	3	3
1	1	3	1		4	3	1	3
1	4	3	3		1	4	3	4
3	3	3	1		4	3	4	1
4	2	3	3		1	4	1	1
2	4	3	3		2	3	4	3
4	3	3	4		2	3	4	3
3	4	1	3		1	4	1	3
1	1	1	4		1	2	3	3

1=*leu*$^{-}$ *cdc37*$^{+}$ 2=*leu*$^{-}$*cdc37*^{ts} 3=*leu*$^{+}$*cdc37*$^{+}$ 4=*leu*$^{+}$*cdc37*^{ts}

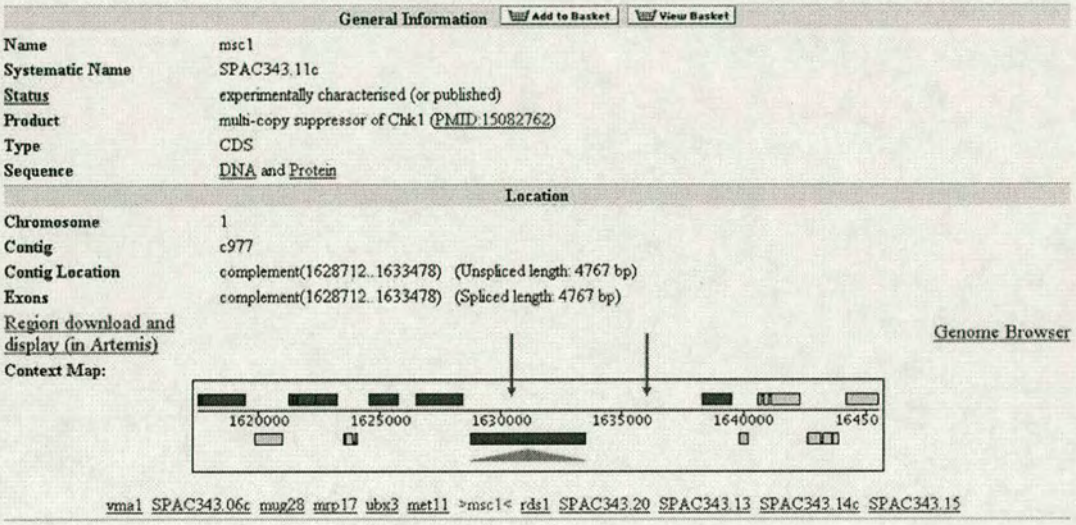
4.4 Investigation of candidate gene in J222

After transformation of J222, 7 strains with plasmid-conferred 5FOA resistance were identified. Of these, five were from *Hin*dIII library and two from the *Bam*HI library. All 5 samples were transformed back into the mutant to test whether they could rescue. The result show only plasmid J222-H1 can rescue the strain.

The sequence results show that the insert was located in chromosome 1 on contig c977 and contig location is between 1632195 and 1636162 (Figure 4.9). Only one big gene, *msc1* locates in the region. *msc1* is a multicopy suppressor of conserved protein kinase *chk1*. It was reported to be chromatin associated and has a histone deacetylase activity (Ahmed et al., 2004). The insert that I isolated is N-terminal truncated version of the *msc1* gene which retains 3/4 region of the whole gene.

The next experiment was to transform the plasmid carrying the authentic *msc1* gene into J222 to check whether it can rescue the mutant. The plasmid pSP1-Msc1HA which was constructed in the pSP1 vector was used to transform J222 mutant. pSP1-Msc1 contains the full length of *msc1* with HA tag in C-terminal. The transformed cells were grown on EMMG+leucine for 4 days. Single colony was picked to test growth on EMMG+leucine+ 5FOA for 2 days. The result shows that the full length of *msc1* can rescue the mutant (Figure 4.10). It indicates that *msc1* is a possible candidate gene for J222.

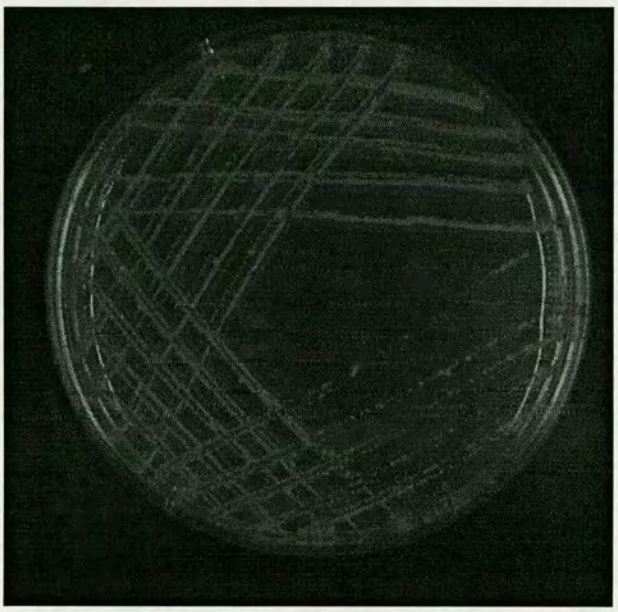
Figure 4.9 Location of the J222-H1 insert sequence in *S. pombe* genome



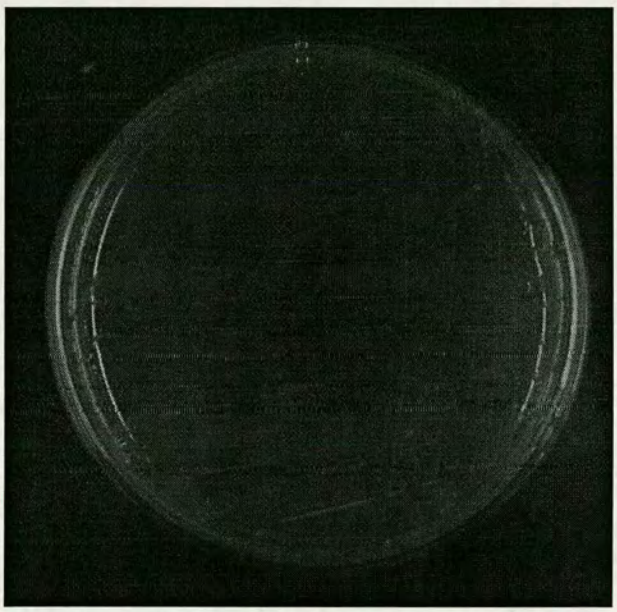
The BLAST result shows which gene in the isolated fragments in J222.

The BLAST result shows which genes are present in the isolated fragment.. The two blue arrows indicate the position of the ends of the J222-H1 insert

Figure 4.10 Transformation of the authentic gene into the mutant



J222 pSp1-MscHA on 5FOA



J222 on 5FOA

The transformed cells were picked and streaked on EMMG+ura+5FOA and incubated at 28 °C for 2 days

To test whether *msc1* is synthetically lethal with *cdc37-681*, I crossed the *msc1* deletion strain ED1577 in which *msc1* gene was deleted and replaced by kan^R resistance gene (*msc1*::kan^R *leu1-32 ura4-D18 ade6-210*) with *cdc37ts* strain ED1538 (*cdc37-681 leu1-32 ura4-D18*). Spores were plated on YE plates and incubated at 28 °C for 4 days. 80 spores were picked to incubate on YE and then replicated onto YE+G418 at 28°C and YE plates at 36 °C. They were scored for G418 resistance and examined under microscope for *cdc37ts* phenotype.

The results show that 14 of 80 spores belong to genotype 4 (kan^R $cdc37^{ts}$) (Table 4.3). This indicates that the cells which have *msc1* defect in combination with *cdc37ts* are alive. This experiment suggests that the synthetic lethal mutation of J222 is not in *msc1*.

msc1 encodes a non essential gene, which was isolated as a multicopy suppressor of *chk1* defective in the DNA damage checkpoint pathway in fission yeast. Study shows that Msc1 possess three PHD fingers, domains commonly found in proteins that influence the structure of chromatin. Cells lacking Msc1 have a dramatically altered histone acetylation pattern. It is supposed to play an important role in regulating chromatin structure and modulate the cellular response to DNA damage (Ahmed et al., 2004). *chk1* encodes a protein kinase, required for cell cycle arrest when DNA damage has occurred. It binds to and phosphorylates Cdc25, which leads to negative regulation of Cdc25 and prevents mitotic entry (Aldea et al., 1994). Overexpression of *msc1* can rescue the *chk1* defect (Ahmed et al., 2004). It is possible that the synthetic lethal mutation is *chk1* in J222.

Table4.3 The genotype of cross by random spores

msc1::kan^R leu1-32 ura4-D18 ade6-210 x *cdc37-681 leu1-32 ura4-D18*

2	2	1	2		1	3	4	2
2	3	3	2		3	2	2	3
2	2	1	3		2	3	2	1
2	4	3	2		3	3	2	1
1	2	4	1		4	2	1	3
3	4	1	1		1	4	3	1
4	1	4	3		2	1	1	4
2	3	2	3		3	2	4	2
4	3	1	1		4	1	1	3
2	3	2	1		3	4	1	4

1=*kan^{S}cdc37$^+$* 2=*kan^{S}cdc37^{ts}* 3=*kan^{R}cdc37$^+$* 4=*kan^{R}cdc37^{ts}*

Table4.4 The genotype of cross by random spores

chk1:: ura4-D18 leu1-32ade6-210 x *cdc37-681 leu1-32 ura4-D18*

3	3	3	3		3	3	3	3
4	2	2	1		4	4	2	4
3	3	2	2		4	3	2	3
2	2	4	2		4	2	2	3
2	4	3	3		3	4	4	2
3	2	2	4		1	2	2	4
1	2	4	1		4	2	4	4
4	1	1	2		4	4	2	2
4	2	1	2		1	4	2	1
1	2	2	4		1	1	1	4

1=*ura*$^{-}$ *cdc37*$^{+}$ 2=*ura*$^{-}$*cdc37*^{ts} 3=*ura*$^{+}$*cdc37*$^{+}$ 4=*ura*$^{+}$*cdc37*^{ts}

I crossed the *chk1* deletion strain ED1023 in which *chk1* gene was deleted and replaced by *ura4* gene (*chk1*::*ura4-D18 leu1-32 ade6-210)* with *cdc37ts* strain ED1538 (*cdc37-681 leu1-32 ura4-D18*). Spores were plated on YE plates and incubated at 28 °C for 4 days. 80 spores were picked, incubated on YE and then replicated onto EMMG+leucine 28°C and YE plates at 36 °C. They were counted for ura^+ and examined under microscope for *cdc37ts* phenotype.

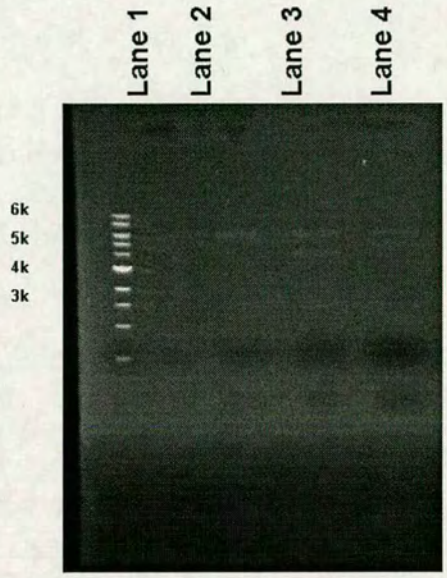
23 of 80 spores belong to genotype 4 (Table 4.4). It shows that the cells with *chk1* defect and *cdc37ts* double mutations were alive. It suggests that the synthetic mutation in J222 is not in *chk1* either.

4.5 Investigation of candidate gene in J218

After transformation of J218, 13 strains with plasmid-conferred 5FOA resistance were identified. Of these, 8 were from *Hin*dIII library and 5 from the *Bam*HI library. The restriction enzyme analysis was carried out on the 13 plasmids. All samples were retransformed back into the mutant to test whether they can rescue. The result shows only plasmid J218-H3 and J218-B4 (Figure 4.11) can rescue the strain.

The genomic region which was spanned by the J218-H3 insert was located on chromosome 2 in contig pJ5566 and contig location is between 864543 and 868285 (Figure 4.12). There are several genes in the region. The most interesting one is the gene *nak1*. The insert covers the entire region of the *nak1* gene. *nak1* encodes an essential protein kinase. It belongs to the serine/threonine protein kinase family, and plays an important role in the regulation of cell polarity, growth and division.

Figure 4.11 Restriction enzyme analysis of plasmids were recovered in J218 mutant



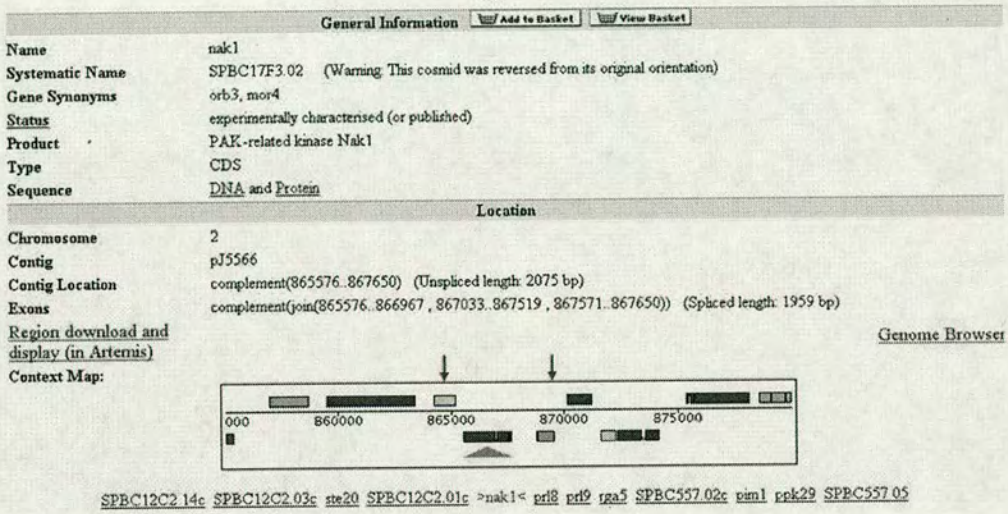
Lane1: 1kb ladder DNA maker

Lane2: empty vector was digested by *Hin*dIII

Lane3: J218-H3 was digested by *Hin*dIII

Lane4: J218-B4 was digested by *BamH*I

Figure 4.12 Location of the J218-H3 insert sequence in *S. pombe* genome

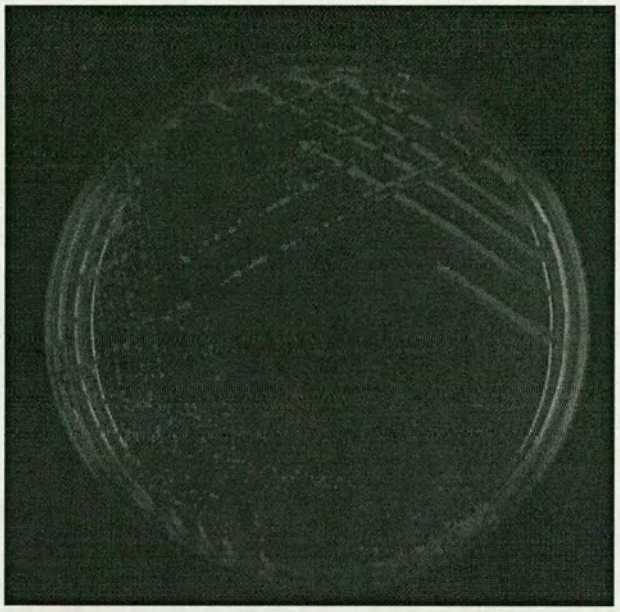


The BLAST result shows which genes are present in the isolated fragment. The two blue arrows indicate the position of the ends of the J218-H3 insert

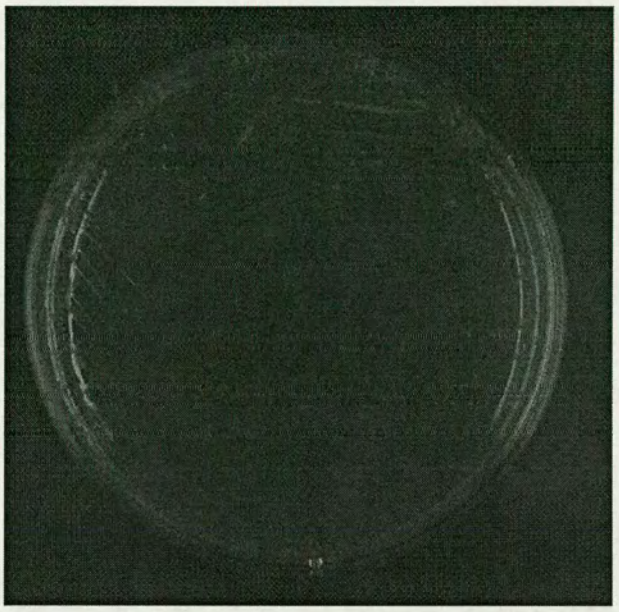
(Leonhard and Nurse, 2005).The other gene is *pr18*; it is non-coding RNA. The insert also covered the very small part of gene, named SPBC12C2.01c. No homologous gene or gene function has been reported about it.

I was sent the plasmid pREP4x-*nak1* which carries the full length of *nak1* and based on *ura4* marker (Leonhard and Nurse, 2005). It could not be used to test J218 mutant directly, because the original synthetic lethal mutant was kept alive by *ura4* based plasmid expressing *cdc37* protein. Construction of a leu-base plasmid for expression *nak1* gene was needed. To make it, a fragment containing part of the vector and *nak1* were cut from pREP4x-*nak1*. pREP41 was treated with *Dra*III which cuts the position of 6368 and *Sal*I at 3679. The big fragment containing *nak1* was ligated to the correspondence restriction site in pREP41. After transformation of pREP41-nak1 into J218, cells were plated on EMMG+leucine to let *ura*$^+$ clones to grow up. Then they were tested on EMMG+leucine+ 5FOA for 2 days at 28 °C. The transformed cells show 5FOA resistance on the plate (Figure 4.8). It indicates that full length of *nak1* can rescue the mutant. *nak1* is a possible gene candidate which is synthetic lethal with *cdc37-681*.

4.13 Transformation of the *nak1* into the mutant



J218 with pREP41-nak1 on 5FOA



J218 on 5FOA

The transformed cells were picked to plated on EMMG+ura+5FOA at 28 °C for 2 days

nak1 encodes an essential protein kinase. *orb3* is an alternative name for *nak1*. The Nak1 kinase is essential for polarizing the actin cytoskeleton and needed for cell separation in fission yeast. It is required for F-actin to be concentrated at the cell poles during interphase and to regulate events in late cytokinesis (Leonhard and Nurse, 2005).

To test whether *nak1* is synthetically lethal with *cdc37ts*, the strategy will be to construct the double mutants and test for synthetic lethality with *cdc37ts*. The easiest way would be to cross a *nak1* deletion strain with *cdc37-681*. But *nak1* is an essential gene; the deletion of *nak1* would induce the death of the cells. *orb3/mak1* temperature sensitive strains have their own lethal phenotype at the restrictive temperature of 36 °C(Figure4.13). They can be used to cross with *cdc37-681* strains. The table shows the phenotypes of the expected tetrad types. The tetratype asci are the most important. Each should contain one spore with *cdc37-681* phenotype; one spore with *orb3* phenotype; one spore wild type spore; the other one will be dead if synthetic lethal interaction happens (Table 4. 5). If *nak1* shows synthetic lethality with *cdc37-681*, the *orb3* and *cdc37* double mutant will be dead at permissive temperature.

To carry out in practice, both *orb3-167 and orb3-35/2* were used to cross with *cdc37 ts* strain. Asci were dissected from each cross and spores were incubated at 28 ℃ until the colonies formed. They were then replicated on YE and incubated at restrictive temperature. The cells were examined to identify *orb3* and *cdc37* phenotypes under microscope.

Table4. 5 The expected genotype and phenotype of the progeny in tetrads from *orb3-167* x *cdc37-681*

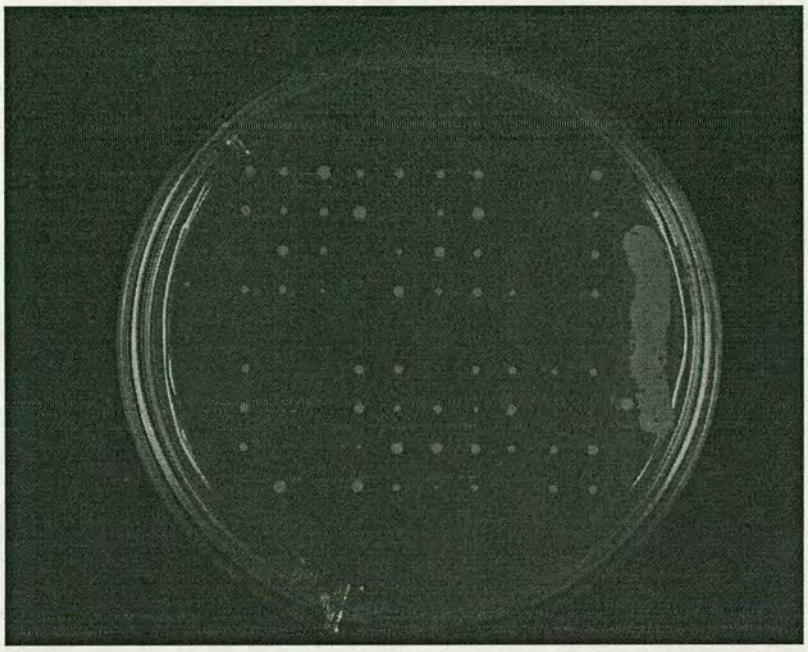
Type of Tetrad	Genotype of the progeny	Phenotype of the progeny
Parental ditype	2 *cdc37* 2 *orb3* spores	2 *cdc37* 2 *orb3* spores
Tetratype	1 wildtype 1 *cdc37* 1 *orb3* 1 *orb3 cdc37*	1 wildtype 1 *cdc37* 1 *orb3* 1 double mutant phenotype
Non-parental ditype	2 wild type 2 *orb3 cdc37*	2 wild type 2 double mutant phenotype

15 of 20 asci were successfully dissected in the cross of *orb3-167* and *cdc37-681* strains. In most asci, 4 viable spores presented. The segregation pattern within each asci was deduced after examining the phenotype of each spore. 10 of 15 asci belonged to tetratype. In these asci, one spore exhibited wild type phenotype; two exhibited *orb3* phenotype 36 ℃ and one exhibits *cdc37* phenotype at 36 ℃. 3 asci belonged to parental ditype, which have two *orb3* phenotype and two *cdc37* phenotype spores. one ascus belonged to non parental ditype, which have two wild type and two *orb3* phenotype spores (Figure4.13). The cross of *orb3-35/2* with *cdc37-681* showed similar results (data was not shown).

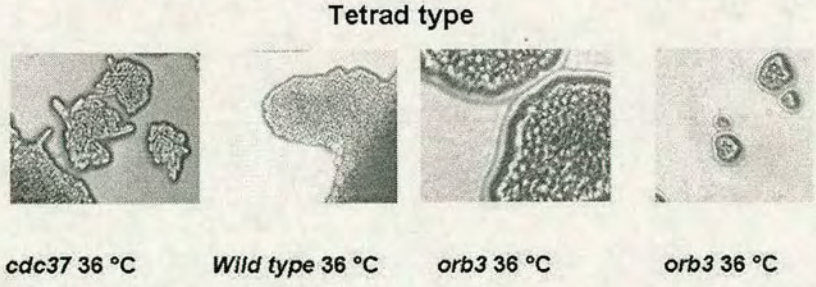
In tetratype asci, two spores exhibited *orb3ts* phenotype. It is likely that the genotype of one "orb3" spore is *orb3ts* mutant and the other "orb3" spore is the double mutant *orb3ts cdc37-681*. To identify which "orb3" spore is the double mutant, the two strains are backcrossed with wild type strain. Three kinds of spores *orb3*, wild type and *cdc37* will be produced when the double mutant is backcrossed with wild type. Only two kinds of spores *orb3* and wt will appear when the *orb3* mutant is backcrossed with wild type.

The two "*orb3-167*" tetratype spores from one ascus were crossed with wild type strain ED0862. Successful crosses were incubated in Helicase to release the spores. Spores were plated on YE 28 °C and replicated to 36 ℃ to check under microscope. The results show only 3 kinds of phenotype *orb3*, wild type, *cdc37* can be observed in both of the two "orb3" spore cross. The experiment shows that the double mutant is alive and exhibits the phenotype of *orb3-167*. The same result comes out from the

Figure 4.13 Tetrad dissection of the cross *orb3-167 x cdc37-681*



The spores were incubated on YE at 28 °C for 4 days



The spores were incubated on YE at 28 °C for 4 days, and then replicated on YE at 36 °C overnight. Phenotype of spores were examined under microscope

cross of *orb-35/2* and *cdc37-681*. It suggests that the synthetic mutation in J218 is not in *nak1*, although it can be rescued by the plasmid contain the *nak1* gene fragment.

4.6 Summary of the chapter

Genomic libraries were used to transform all 10 synthetic lethal mutants. Plasmids which can rescue the mutants were isolated from 5 strains, J05, J06, J222, J218 and J322. For the other strains, I failed to get the plasmid after transformations or plasmids were eliminated for not rescuing the mutants very well during retransformation test. The same fragments have been isolated from two different mutants J05 and J06. Sequences were run through a DNA BLAST search at the Sanger Centre database. *wis4*, encoding a MAP kinase kinase kinase involved in the stress-responsive signal transduction pathway was identified as a candidate gene that rescues the mutants. By crossing *wis4* and a closely related kinase *win1* deletion strains with *cdc37-681*, it was proved that *wis4* and *win1* are not synthetic lethal with *cdc37-681*. From J222, *msc1,* a multi-copy suppressor of *chk1,* was identified as a possible candidate gene which can rescue J222. However, the results of crossing *msc1* and *chk1* deletion strains with *cdc37-681* show neither *msc1* nor *chk1* is synthetically lethal with *cdc37-681*. The genomic fragment containing protein kinase *nak1* was isolated from J218. *nak1* encodes an essential protein kinase and plays a role in the regulation of cell polarity, growth and division. But two *nak1ts* mutants, *orb3-167* and *orb3-35/2* do not show synthetic lethality with *cdc37-681* by cross.

Through the results, I know that the gene isolated from the plasmid can rescue

the mutants, but they were unlikely to be the actual gene in the chromosome which was synthetic lethal with *cdc37-681* in the mutant as I initially expected. The possible explanation will be that the candidate genes that I isolated were suppressors of the actual synthetic lethal gene in the mutant. There are several mechanisms by which suppressors are obtained during the plasmid based screening. Dosage suppressors may be isolated when they are over expressed and the gene products can stabilize the mutated protein and help protein to fulfil its function. The most common suppressor will appear as the interaction suppressor. If two proteins can interact physically, mutating one of them might disrupt the interaction. The theory of function of bypass suppressor is similar to interaction suppressor. If mutation of the gene can block the pathway which rely on the function of the gene, bypass suppressor will activate alternative pathway to replace the function of the mutated gene. Although it was hard to tell which kind of the suppressor that the candidate gene will be, the fact is that it was easy to isolate suppressors during library screening.

To test whether candidate genes were synthetic lethal with *cdc37-681*, usually deletion strains were used to cross with *cdc37ts* strain. Whether or not the double mutants appeared to be alive were observed to test the synthetic interaction of the two genes. I imagine that the specific mutation in the specific gene may show synthetic lethal interaction with *cdc37ts*, while the synthetic lethal interaction might not happen when the the strain with deletion of this gene was crossed with *cdc37ts*. For example, *atb2* encoding an alpha-tubulin gene, the mutation in *atb2* can give a ts phenotype, although the gene can be deleted without loss of viability (Radcliffe et al., 1998). In our case, it is possible that the mutation in *wis4, win1, msc1, chk1* of the mutant may be synthetic lethal with *cdc37ts*. But the strains with deletion version of genes do not show synthetic lethality with *cdc37ts* as we had observed.

5.1 General introduction

In the previous chapter, I described that each of 10 synthetic lethal mutants was transformed by genomic libraries. Plasmids that rescued 5 of the mutants were recovered and sequenced. Sequences were run through a DNA BLAST search at the Sanger Centre database. *wis4*, *msc1*, *nak1* were identified as candidate genes that rescue the various mutants. Further experiments shows that *wis4*, *msc1*, *nak1* were not synthetic lethal with *cdc37-681*. Cdc7 is a protein kinase essential for septation and cell division. Results are present in this chapter, suggesting that Cdc7 is of special interest and is a possible client protein of Cdc37.

5.1 Transformation of *S. pombe* genomic libraries into strain J322

J322 is one of the synthetic lethal mutants from the screening. It was maintained by a wild type copy of *cdc37* on the *ura4*$^+$ based vector pREP82. When the mutant is transformed with a genomic library of plasmids containing *leu*$^+$ selective marker, all successful transformants will grow on EMMG+ura medium. After replication onto EMMG+ura+5FOA, cells can only survive and grow on EMMG+ura+5FOA, if the gene carried by *leu* plasmid is able to compensate the loss of function of the J322 chromosomal mutation.

To carry out the transformations, the electroporation procedure was applied as described in Chapter 3. In the final step, 200 μl aliquots of each diluted sample were plated onto 4 EMMG+uracil plates. The plates were incubated at 28 ℃ for

four days to allow colonies to form. There were around 3000-4000 colonies per plate, which was enough to cover at least one genome equivalent during one transformation. The plates were then replicated onto EMMG+ura+5FOA at 28 ℃. This selects the colonies which have a *leu* plasmid carrying a gene able to rescue the mutant which are able to lose the *ura* plasmid carrying wild type *cdc37*. In this screen, 15 colonies can survive on EMMG+ura+5FOA. 5FOA resistant colonies were picked to recover the plasmid from them.

A total of 8 strains with plasmid-conferred 5FOA resistance were identified in 5FOA screen after transformation of J322. Of these, five were from *Hin*dIII library and three from the *Bam*HI library, but no plasmids were recovered from *Spe*I library. All plasmids were transformed back into J322 to test whether they could rescue. It was found that the only plasmid J322-H7 can rescue J322 mutant successfully.

5.2 Sequencing and analysis of the genomic insert in J322-H7

To investigate which gene or gene fragment is involved in rescuing J322, sequencing the genomic insert is the most direct way. The sequencing was done from both ends by using the primer M13-21 (forward) and M13+21 (reverse) universal sequencing primers. The "clean" (vector-free) insert sequences were run through a DNA BLAST search at NCBI database (The National Center for Biotechnology Information). It allows identification of the genomic region that the insert spanned. Further information was accessed through the Gene Database at website http://www.genedb.org/genedb (Figure 5.1).

The plasmid J322-H7 was isolated from the synthetic lethal mutant J322 and has the ability to rescue the original mutant. The genomic region which was spanned by the insert was located on contig pJ5566 with contig coordinates 1607158 and 1612757 (Figure 5.2). There are several genes in the region. The insert includes the most of the *cdc7* gene. *cdc7* encodes a protein kinase essential for cell division, which plays a key role in initiation of septum formation and cytokinesis (Fankhauser and Simanis, 1994). It belongs to the serine/threonine protein kinase family. A small gene called *ppk24* was also present in the J322-H7 insert. It was predicted as protein kinase from its sequence. Little knowledge exists about the function of *ppk24.* A small part of a small gene *spn7* is present in this region. No paper describes the function of this gene or *ppk24*. The possible function of *spn7* which is predicted from its sequence is that it may be involved in cell division and act as a sporulation specific protein.

Figure 5.1 Sequence alignment of J322-H7 sequence derived using M13-21 forward primer with *S. pombe* database

```
Query:       13 ATCGTCAGTAGCCAAAGCTTTCAGCATGGCTTTGGTCAAGGGCTCTAGTGAATCATTTAC 72
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1607158 ATCGTCAGTAGCCAAAGCTTTCAGCATGGCTTTGGTCAAGGGCTCTAGTGAATCATTTAC 1607217

Query:       73 AACCATCGTACAGATATCATTTTTTGGAATGTAATCCTGTTGTCTCAGTAATTTCCATAT 132
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1607218 AACCATCGTACAGATATCATTTTTTGGAATGTAATCCTGTTGTCTCAGTAATTTCCATAT 1607277

Query:      133 TCCTTCTACGCCAACAAAAACAAAGTCACGGTTCGTTCCATAGTCCTCTTTAATAAATAA 192
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1607278 TCCTTCTACGCCAACAAAAACAAAGTCACGGTTCGTTCCATAGTCCTCTTTAATAAATAA 1607337

Query:      193 TAAAAGCGAATTCAGACCATTTGAGCTCAAAAACATTTGTAATGTCAAAGCAGAAGTTCT 252
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1607338 TAAAAGCGAATTCAGACCATTTGAGCTCAAAAACATTTGTAATGTCAAAGCAGAAGTTCT 1607397

Query:      253 ATACATCTGTTGAATAAAGATAGCACTTTCATATCTAAACTCAAAAGAGTGTTCACGATT 312
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1607398 ATACATCTGTTGAATAAAGATAGCACTTTCATATCTAAACTCAAAAGAGTGTTCACGATT 1607457

Query:      313 AGAGAATGACAACATAAGCGGTAAACCTCCAGCAAAGCAAACTTTTTGTAAAGTTGTGTG 372
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1607458 AGAGAATGACAACATAAGCGGTAAACCTCCAGCAAAGCAAACTTTTTGTAAAGTTGTGTG 1607517

Query:      373 ATCATCGAATGCAACCTAACGTTAGTAAAATCACGCATTAAATATCCTTACAGTGTTAAT 432
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1607518 ATCATCGAATGCAACCTAACGTTAGTAAAATCACGCATTAAATATCCTTACAGTGTTAAT 1607577

Query:      433 GAGCTTTAATAACAGCAATTGAACATCAGGCGTTTTTATTTCGCGCAATGTCTCTA 488
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1607578 GAGCTTTAATAACAGCAATTGAACATCAGGCGTTTTTATTTCGCGCAATGTCTCTA 1607633
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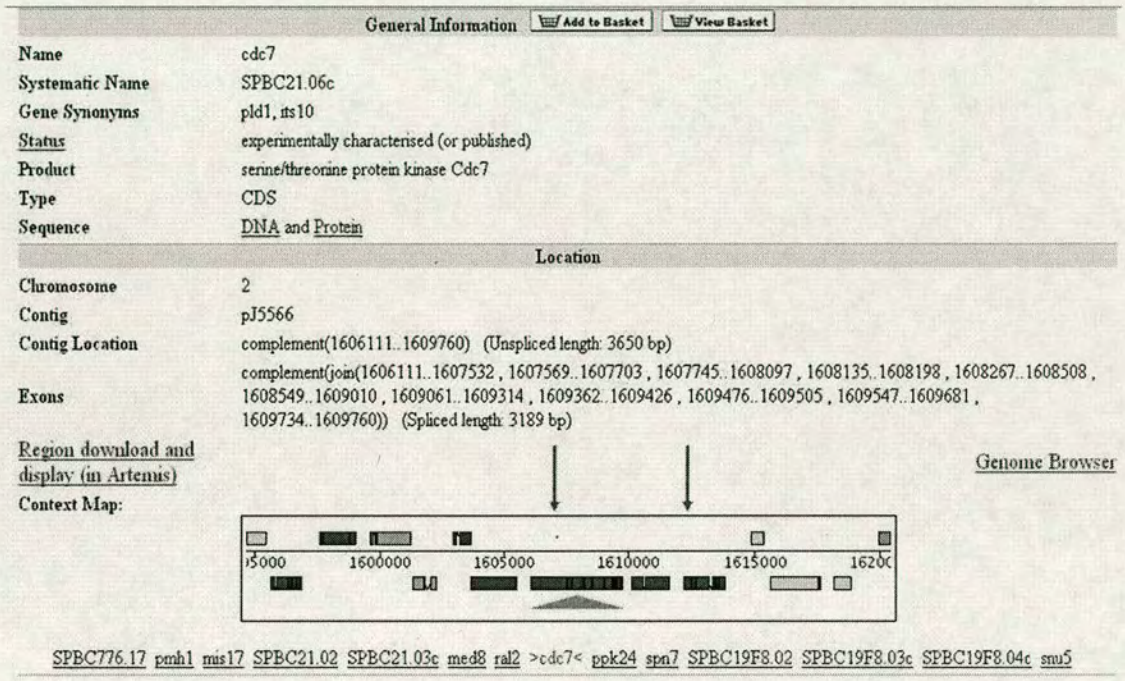
The top line is the end of sequence of J322-H7 derived by sequencing using the forward primer. The lower line is the chromosomal sequence in the database.

Figure 5.2 Location of the J322-H7 insets sequence in *S. pombe* genome



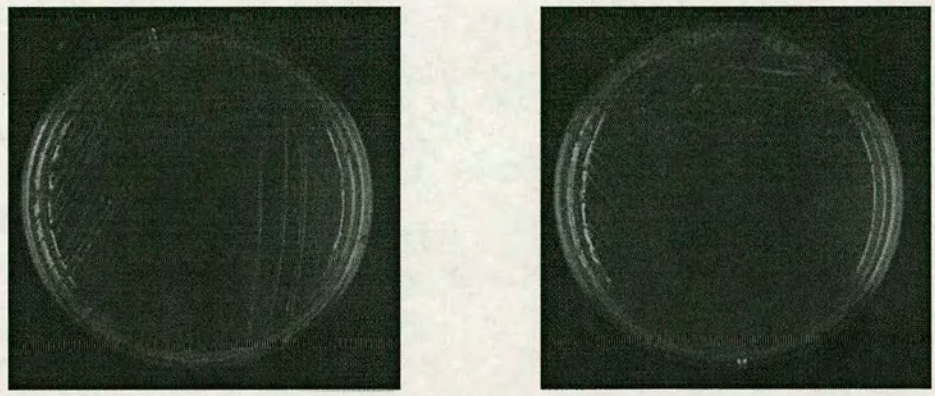
blue arrows indicate the position of the ends of the J322-H7 insert

5.3 Identification of the rescuing gene in J322-H7

Possible candidate genes were uncovered by sequencing plasmid J322-H7. The genomic DNA insert contains more than one gene, and the next step is to distinguish which gene is responsible for rescuing the mutant. Furthermore the plasmid J322-H7 has a truncated version of *cdc7* gene. I wondered whether the intact version of the gene is able to rescue the mutant.

The J322-H7 insert covers the almost the full length of the *cdc7* gene. An N-terminal truncation of Cdc7 has been reported to rescue *cdc7ts* mutation (Fankhauser and Simanis, 1994). Therefore Cdc7 protein may be responsible for rescuing the J322 mutant.The plasmid pREP41-cdc7 which expresses full-length Cdc7 was obtained from Dr Vienurs Simanis and tested for its ability to rescue the J322 defect. After transforming J322, cells were plated on EMMG+Ura to let leu^+ clones to grow up. Then they were tested on EMMG+Leu+ 5FOA for 2 days. The transformed cells show 5FOA resistance on the plate (Figure 5.3). It suggests that the full length *cdc7* can rescue the mutant. *cdc7* is therefore a possible gene candidate which is synthetic lethal with *cdc37-681*.

Figure 5.3 Transformation of the *cdc7* into the mutant



J322 with pREP41-cdc7 on 5FOA J322 on 5FOA

The transformed cells were plated on EMMG+ura+5FOA at 28 °C for 2 days, J322 were plated on EMMG+ura+leu+5FOA at 28 °C for 2 days

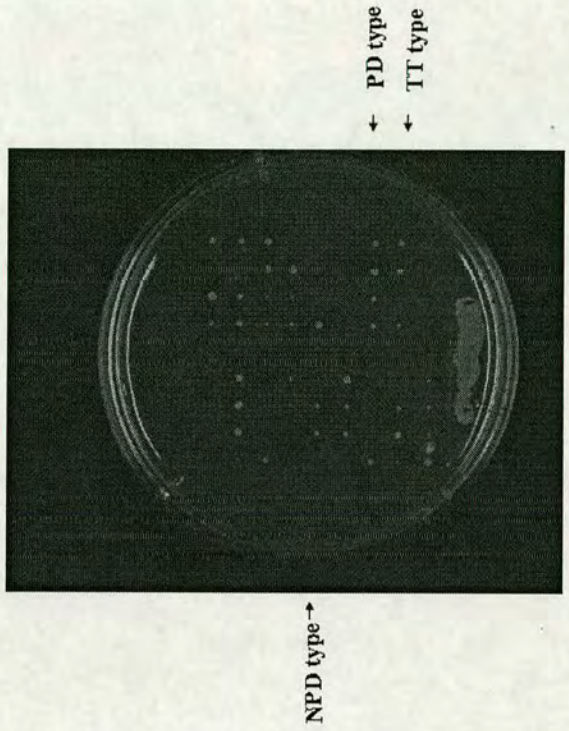
5.4 Genetic analysis to test whether the isolated gene is synthetically lethal with *cdc37ts*

To test whether *cdc7* is synthetic lethal with *cdc37*ts, the same strategy will be used for testing *orb3* mutant, described in Chapter 4. *cdc7* is also an essential gene; the deletion of *cdc7* would induce the death of the cells. However a temperature sensitive *cdc7* mutant, *cdc7-24* has its own phenotype at restrictive temperature (Nurse et al., 1976). It can be used to cross with *cdc37-681* strain to test for synthetic lethality.

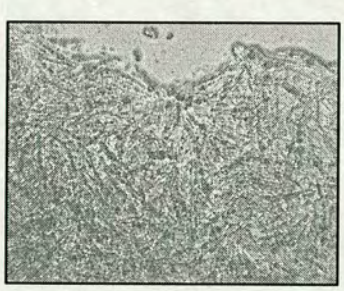
After the cross of *cdc ts* mutant with *cdc37 ts* mutant, asci are dissected and spores are incubated at 25℃ until the colonies form. They are replicated on YE at restrictive temperature to examine *cdc7* and *cdc37* phenotype under the microscope. The asci which exhibit tetratype are of most importance. Each should contain one spore look with cdc37 phenotype; one spore with cdc7 phenotype; one wild type; the remaining spore will be dead if synthetic lethal interaction happens.

In practice, the *cdc7* temperature sensitive strain *cdc7-24* was used to cross with *cdc37-681*. Tetrads were dissected and spore plates were incubated at 25℃ until the colonies formed. The spore colonies were replicated to YE at 36℃ to examine *cdc7* and *cdc37* phenotypes under microscope. 12 of 20 asci were successfully dissected (Figure 5.4). In most asci, 3 viable spores were presented. The segregation pattern of each ascus was deduced after examining the phenotype of each spore. 7 of 12 asci showed tetratype segregation for *cdc7* and *cdc37*. In these asci, one spore exhibited wild type phenotype; one exhibited *cdc7* phenotype at 36 ℃ and one exhibited *cdc37* phenotype.

Figure 5.4 Tetrad dissection of the cross ED1587 (*h⁻ cdc7-24 leu1-32*)x ED1586 (h^+ *cdc37-681 leu1-32 ura4-D18 ade6-210*)

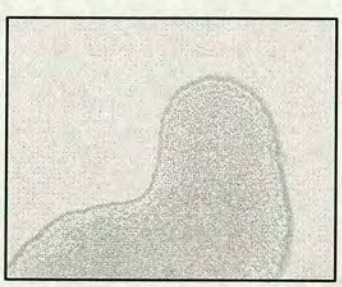


Spores grew on YE at 25 ℃ for 4 days



Cdc7

36 ℃



WT

36 ℃

cdc37

36 ℃

The phenotypes of three spores from one TT type asci were checked under microscope after incubation at 36 ℃. *cdc37* phenotype at 36 ℃.

Three asci belonged to parental ditype, which have two *cdc7* phenotype and two *cdc37* phenotype spores. Two asci belonged to non parental ditype, which have two wild type spores. In the tetratype asci, it was observed that the double mutant was absent at permissive temperature, indicating that defects in both *cdc7* and *cdc37* genes will cause death. So *cdc7-24* is synthetically lethal with *cdc37-681* at the permissive temperature of 25℃.

As further confirmation that "*cdc37*" phenotype and "*cdc7*" phenotype spores are not double mutant, the "*cdc37*" phenotype and "*cdc7*" phenotype spores from separate tetrads are backcrossed with a wild type strain. Only two kinds of spores; wild type and *cdc37* will be produced if "*cdc37*" phenotype is not double mutant. The same situation will happen with "*cdc7*" phenotype spores if they are not the double mutant, only two kinds of *cdc7* and wt progeny will appear when backcrossed with wild type.

The "*cdc37*" phenotype and "*cdc7*" phenotype spores in one ascus were crossed with wild type strain ED0862. Successful crosses were incubated in Helicase to release the spores (Chapter 2). Spores were plated on YE at 25 ℃ to form colonies and then replicated to 36 ℃ to check under microscope. The results show 2 kinds of phenotype: wild type, and either *cdc37* or *cdc7* phenotype can be seen in each case. The experiment shows that the double mutant was not alive. It confirmed that *cdc7-24* is synthetically lethal with *cdc37-681* at permissive temperature.

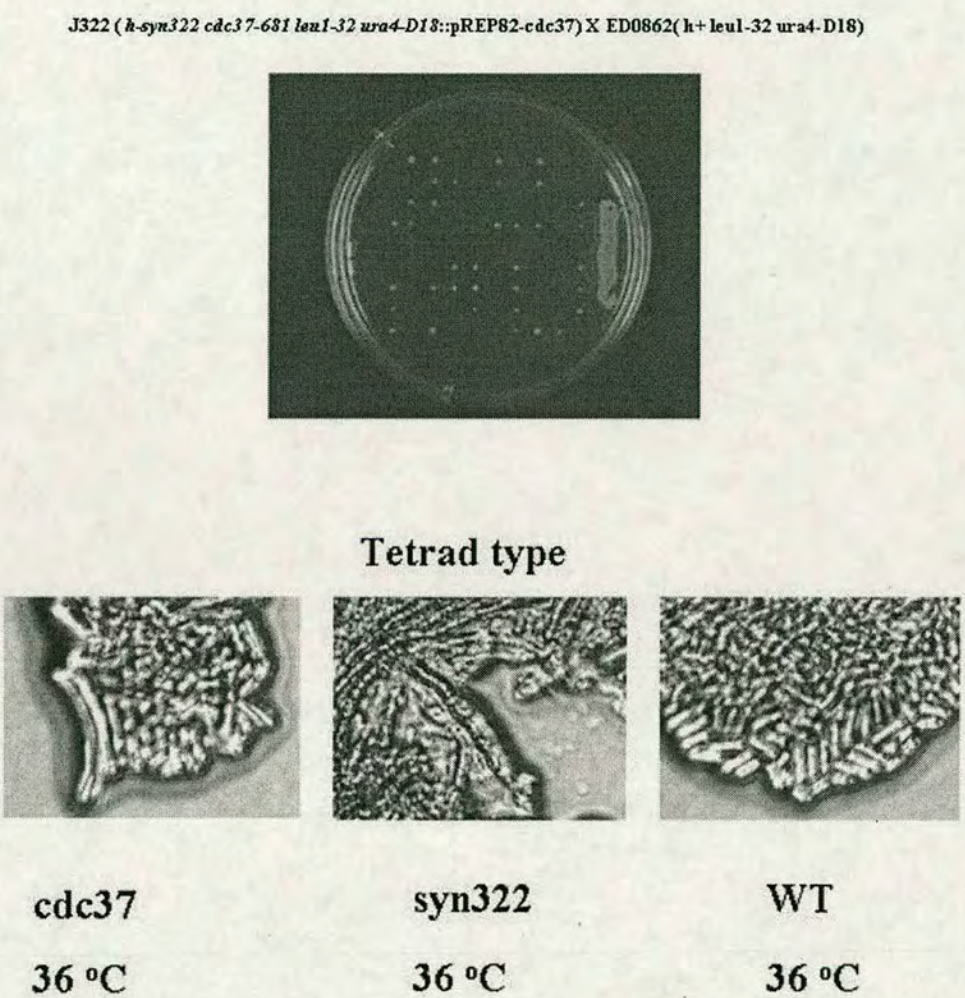
5.5 Outcross of the J322 mutant

To identify whether the synthetic lethal mutation in the chromosome of J322 has its own phenotype, an outcross experiment was carried out.When J322 is crossed with wild type strain, the additional mutation and *cdc37ts* mutation can be separated during chromosome segregation. In tetratype asci, there should be 3 viable spores with typical 3 different genotypes, one wild type, one *cdc37ts*, the other one is the synthetic lethal mutation of J322. The double mutant spores which have combination of *cdc37 ts* and synthetic lethal mutation defects will be dead. In some cases, 4 viable spores will appear in a tetratype ascus, one wild type, one *cdc37ts*, one the synthetic lethal mutation of J322, plus the double mutant rescued by the original plasmid pREP82-cdc37. Spores carrying the plasmid can be identified by the ability to grow on the medium lacking uracil.

In practice, J322 was used to cross with wild type strain ED0862 (h^+ *leu1-32 ura4-D18*). After tetrad dissection, the spores were incubated on YE plates at 28 ℃ for 4 days. Then the spore colonies were replicated onto EMMG+Leu to test for Ura^+. They were replicated to YE at 36 ℃ and examined for cell morphology under microscope.

Asci with 3 viable spores were predominant (Figure 5.6), a result in accord with the above hypothesis. The phenotypes of these spore colonies were checked at high temperature. One of them showed *cdc37ts* phenotype with curved and elongated cells, one looked like wild type and the other one consisted of extremely elongated and lysed dead cells (Figure 5.5). There were asci with 4 viable spores: one with *cdc37ts*, 2 WT spores and one similar *cdc7ts* phenotype spores.

Figure 5.5 Synthetic lethal mutation out of *cdc37ts* background



In a tetrad type asci, there are three viable spores. When they were tested at high temperature, one spore shows WT phenotype, one shows *cdc37-681ts* phenotype, the other shows lysis and elongated phenotype. It indicates that *syn322* mutation

has phenotype of its own in *cdc37* $^{+}$ background, phenotype with curved and elongated cells, two showing wild type, and the other one consisting of extremely elongated and lysed dead cells. When these asci with 4 viable spores were tested for ura^{+}, one of these spores is ura^{+}, it means the spore carried the plasmid and usually exhibits wild type.

All these facts suggest that the synthetic lethal mutation in the chromosome of J322 can be separated out from *cdc37ts* mutation. And it was a *ts* mutation with its distinct phenotype at high temperature.

5.6 Investigation of synthetic lethal mutation in J322

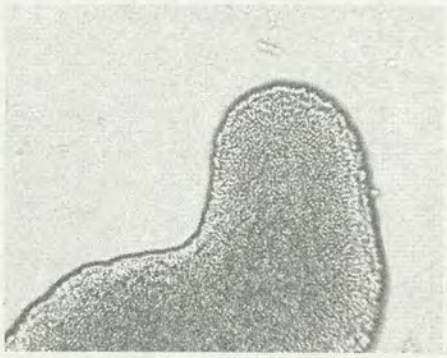
The plasmid containing *cdc7* was isolated from J322. In the meantime, it was verified that *cdc7-24* is synthetic lethal with *cdc37-681* at permissive temperature. The synthetic lethal mutation in J322 was crossed out from *cdc37ts* background. The *syn322* mutant was found to have its distinct phenotype at high temperature. Whether the synthetic lethal mutation in J322 lies in *cdc7* gene is worthy of further investigation. I started with checking the phenotype of *syn322* mutant and the known *cdc7* temperature-sensitive mutant *cdc7-24*.

The cell morphologies of *cdc7-24* and *syn322* mutant were examined after incubation after 24 hours on YE plates at different temperature between 25 °C and 36 °C. At 25 °C, *syn322* mutant and temperature-sensitive mutant *cdc7-24* cells were morphologically wild type in appearance. At 36 °C, the two mutants exhibit the same phenotype with extremely elongated and lysed cells(Figure 5.6). The phenotype of the cell division defect indicates that cells fail to complete the cell cycle and divide, but have the ability to continue growth.

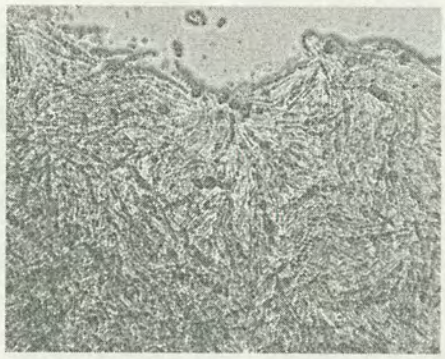
The Cdc7 protein is required for the initiation of septum formation in *S. pombe*, and a mutation in the *cdc7* gene abolishes the ability of cells to produce a septum(Nurse et al., 1976). The distinct phenotype of the *cdc7-24* mutant is that the nucleus of the cells continues to devide while the septum is unable to form at restrictive temperature. After 4 hours incubation at 36 °C, the majority of cells will possess four nuclei without a septum. More nuclei will accumulate in the cell as time goes on. Eventually, 16 or more nuclei are packed in one cell. I wondered whether *syn322* loses the ability to form a septum and accumulates nuclei like the *cdc7-24* mutant.

Syn322 mutant, *cdc7-24* mutant, and wild type strain ED0862 were examined. Samples were taken from cultures at 0 hours and after incubation for 3 hours at 36 °C, fixed with methanol and DAPI (Chapter 2) that can bind the yeast nuclei (Streiblova et al, 1984). Cells were examined and photographed under fluoresecence microscope. At 0 hours, cells in all strains have one nucleus without septum. 3 hours, the cells population of *syn322* and *cdc7-24* possess four nuclei and without septum. (Figure 5.7). It shows that the *syn322* mutant behave the same as the c*dc7-24* at restrictive temperature, which means that *syn322* have the defect in forming the septum and can accumulate nuclei.

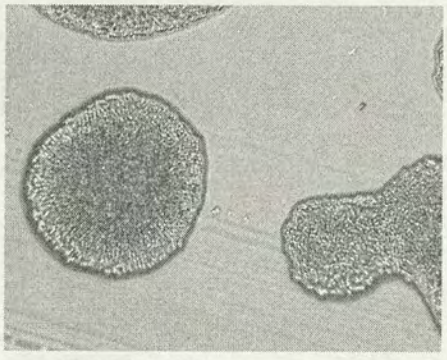
Figure 5.6 Phenotypes of *syn322* and *cdc7-24*



***cdc7-24* 25 ℃**



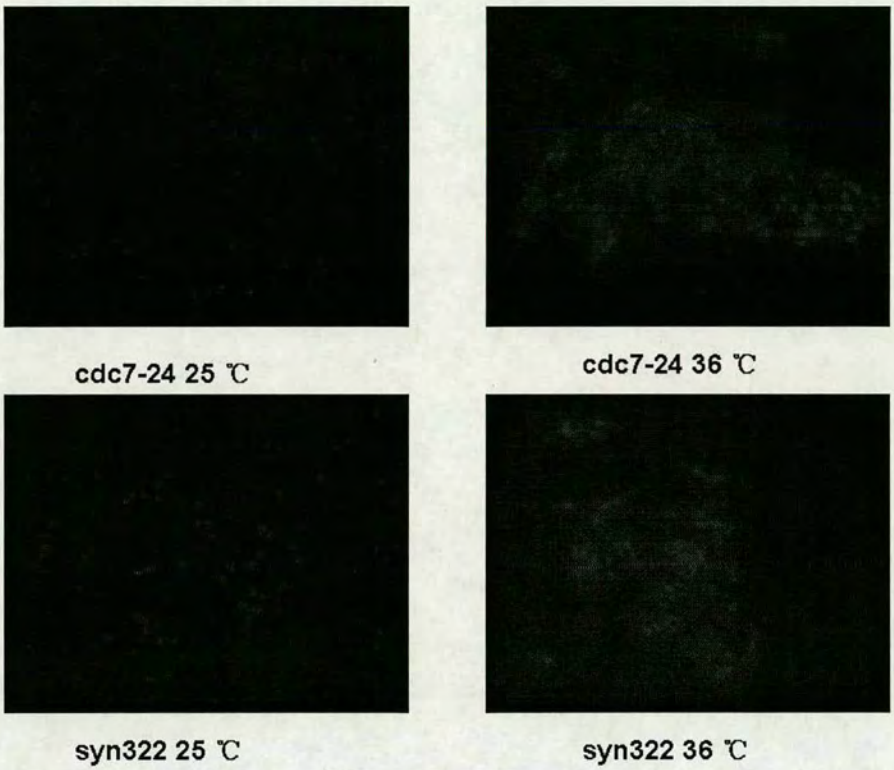
***cdc7-24* 36 ℃**



***syn322* 25 ℃**

***syn322* 36 ℃**

Figure 5.7 DAPI staining of *syn322* and *cdc7-24*



cells were grown in YE over night at 25 ℃ and shited to 36 for 3 hours. Samples were fixed in pre-chilled methanol and stained with DAPI, visualized by fluorescence.

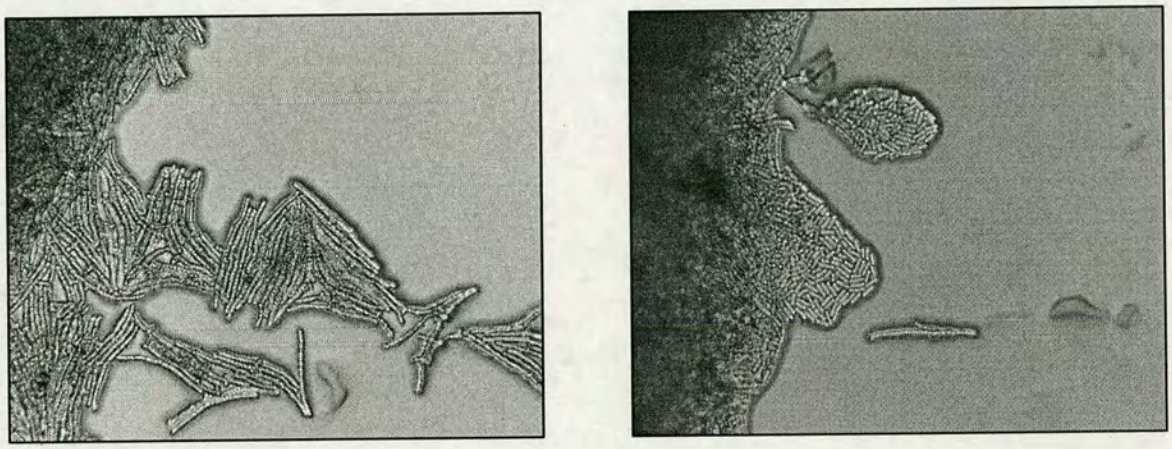
5.7 The syn322 mutation lies in *cdc7* locus

In tetratype asci of J322 outcross, one spore exhibited a phenotype similar to *cdc7-24*. It is likely that the mutation of spore is in the *cdc7* gene. To find out the truth of the hypothesis, the "*syn322"* spores are back crossed with *cdc7-24* strain. If the mutation of the "*syn322*" spore is in *cdc7* gene, all the spores from the cross will exhibit *cdc7ts* phenotype and no wild type spores should appear.

To carry this out in practice, the "*syn322*" progeny from 2 tetratype were crossed with *cdc7-24* strain ED1581. Spores were plated on YE 25 °C until colonies formed. For each cross over 100 spores were picked randomly and replicated to YE at 36 °C. After examining the phenotype of the spores, it was found that all the spores from the crosses showed *cdc7ts* phenotype and no wild type spores were detected. The experiment taken with other data indicates that the synthetic mutation lies *cdc7* gene.

5.8 Investigation of the genetic interaction between *cdc37* and *cdc7*

Figure 5.8 Overexpression of Cdc37 rescues the temperature sensitive defect of *cdc7-24*



cdc7-24 mutant (pREP41) *cdc7-24* mutant (pREP41-cdc37)

The transformants were grown on EMMG+Ura at 25 ℃ for 4 days and replicated on EMMG+Ura at 36 ℃ overnight. Cells were examined under microscope.

From the experiment described in section 5.5, we know that double mutants of *cdc7* and *cdc37* temperature-sensitive mutants are synthetically lethal at permissive temperature, indicating that *cdc7* and *cdc37* interact at genetic level in fission yeast. To further investigate, Cdc37 protein was overexpressed in a *cdc7-24* strain to see whether Cdc37 can help to rescue the *cdc7ts* phenotype at restrictive temperature.

The plasmids pREP41-*cdc7* and pREP41-*cdc37* were used to transform the *cdc7-24* mutant with empty vector pREP41 as the control. The transformants were incubated for 4 days at 25 °C and then replicated to 36 °C. Overexpression of Cdc37 can rescue the temperature sensitive defect of *cdc7-24* (Figure 5.8). The cells were slightly elongated compared to wild type cells but do not show extreme elongation and lysis phenotype as *cdc7-24*. It showed that increasing the Cdc37 protein level can suppress the *cdc7* defect, indicating that Cdc7 may need Cdc37 to fulfil the function and Cdc7 may be a client of Cdc37.

5.9 Studies of Cdc7 protein in *cdc37* temperature-sensitive mutants

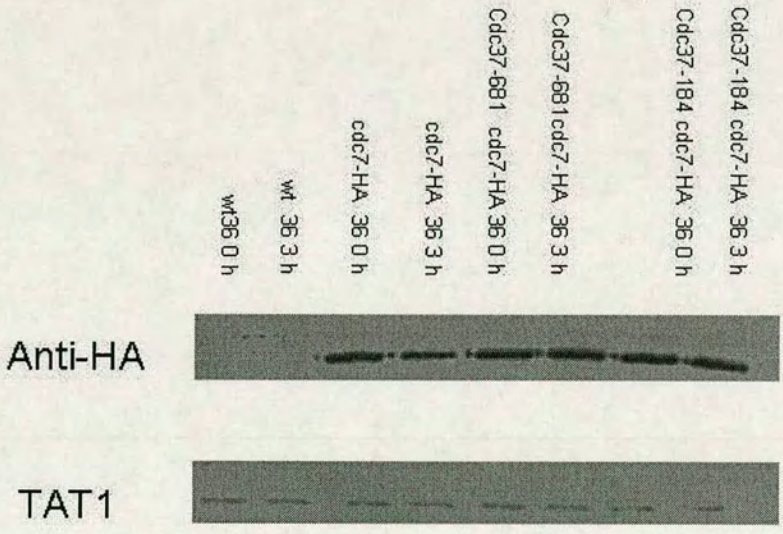
In the previous sections in this chapter, the genetic interaction between *cdc7* and *cdc37* has been proved. To get further information about whether or not the function of cellular Cdc7 protein would be affected by loss of Cdc37, the kinase activity of Cdc7 was assayed in *cdc37ts* strains at restrictive temperature through a time course. The studies of Cdc37 in *S. pombe* showed that around 80% of the cell population of *cdc37ts* mutants were arrested the cell cycle in G2 (Turnbull et al., 2006). Cdc7 kinase activity was also assayed in Cdc2 temperature sensitive strains to test whether loss of Cdc7 kinase activity was a direct effect of Cdc37 defect or an indirect effect of G2 arrest.

Cdc7 kinase activity can be measured by the ability to phosphorylate MBP

(Myelin Basic Protein) *in vitro* (Cerutti and Simanis, 1999). A Cdc7-HA strain was obtained from Dr Simanis, and was used to cross with our lab stock *cdc37-681* and *cdc37-184* mutants to create strains in which the *cdc7* gene was tagged with HA in *cdc37ts* background. Parallel strains were created by the same method in *cdc2ts* mutants. All temperature sensitive mutants and wild type strain ED0862 were cultured in liquid medium at 28 °C to OD_{600}=0.2, then shifted to 36 °C. Samples were taken at 0, 1, 2, 3 hours during the time course. Cdc7 protein was immunoprecipitated from native protein extracts using HA antibody. The kinase activity was assayed in the mixture of substrate MBP and [γ-^{32}p] ATP. The results show that the kinase activity of Cdc7 from wild type strains (Figure 5.10a) and a Cdc2 temperature sensitive mutant; *cdc2-L7* (Figure 5.10b) remains much the same throughout the time course. The other cdc2 mutant *cdc2-33* was tested and the same result obtained (data not shown). The kinase activity was reduced dramatically in both *cdc37ts* strains when the cultures were shifted for 1 hour to the restrictive temperature (Figure 5.10 a,b), indicating that Cdc7 kinase activity was reduced swiftly in *cdc37ts* mutants.

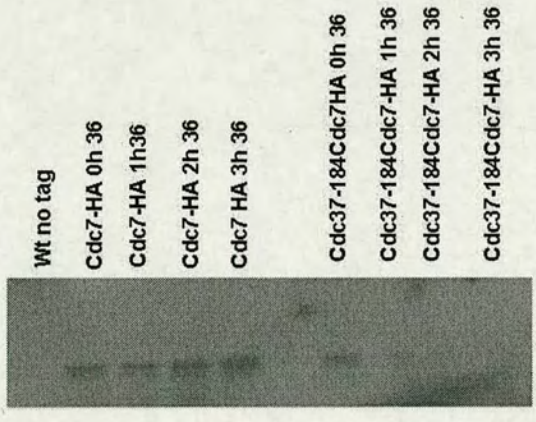
Cdc7 protein levels were also assayed to determine whether reduced Cdc7 activity was due to lower Cdc7 protein in *cdc37* temperature sensitive mutants, as Spc1 protein level in *S. pombe* temperature sensitive mutant *cdc37-681* was reduced (Tatebe and Shiozaki, 2003). Denatured extracts were made from the samples of *cdc37-184* and *cdc37-681* mutants and wild type strain which were taken 0 and 3 hours from cultures at permissive or restrictive temperature. Western blot analysis was done with anti-HA antibody that recognises Cdc7-HA and TAT1 antibody as loading control. The result shows that Cdc7 protein levels did not change over the time either in *cdc37ts* or wild type cells at 36 °C (Figure 5.9). This indicates that the reduction of Cdc7 kinase activity in *cdc37ts* strains was not caused by reduction in Cdc7 protein level.

Figure 5.9 Cdc7 protein levels in cdc37ts mutants and wild type strains



Denatured extracts were made from *cdc37ts* mutants at permissive and restrictive temperature at 0 and 3 hours

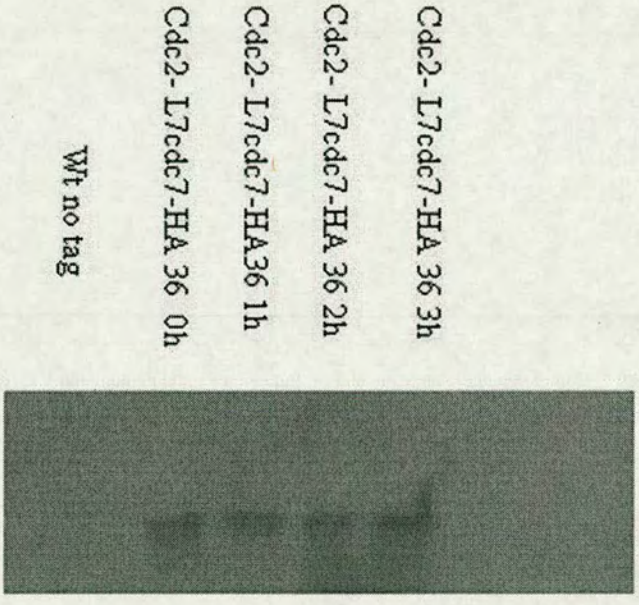
Figure 5.10.a Cdc7 kinase activity in *cdc37ts* mutants



In wt In *cdc37-184*

Cdc7 kinase activity was assayed in *cdc37ts* mutants at permissive and restrictive temperatures.

Figure 5.10.b Cdc7 kinase activity in *cdc2ts* mutants



Cdc7 kinase activity was assayed in *cdc2ts* mutants at permissive and restrictive temperatures.

5.10 Cytological observations on Cdc37 and Cdc7 mutants

Research shows that several proteins are essential for initiation of septum formation in fission yeast, such as Cdc11, Cdc14, Cdc16, Cdc7, Plo1 and Spg1(Simanis, 2003). Cdc16 acts as a negative regulator of the process. The loss of function of Cdc16 results in production of multiple septa without cell cleavage (Fankhauser et al., 1993), while Plo1 and Spg1 act as positive regulators. Overexpression of either protein will induce septum formation in G1 or G2 arrested interphase cells (Mulvihill et al., 1999b) (Stepanova et al., 2000). Cdc7 encodes a protein kinase, and its activity is required for the initiation of septum formation. It was found that Cdc7 kinase activity is present and stable at all stages of the cell cycle by measuring the activity in arrest-release of *cdc25-22* mutants (Cerutti and Simanis, 1999). My experiment of the previous section shows that the Cdc7 kinase was unchanged in G2 arrested *cdc2ts* mutants, which confirms the conclusion.

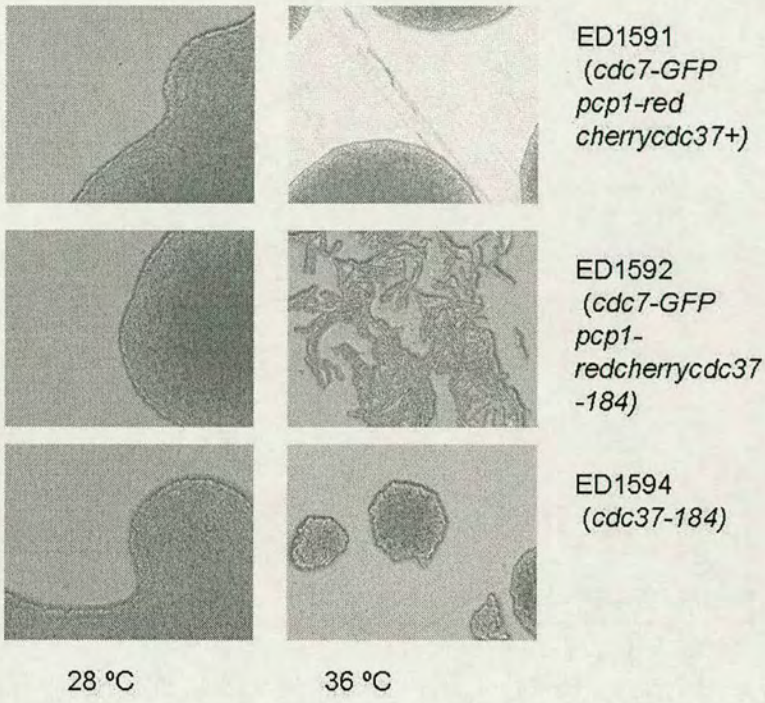
The synthetic lethal interaction between *cdc7* and *cdc37* was described in previous sections. At the same time, Cdc7 kinase activity was dramatically reduced in *cdc37ts* mutants. These results suggest that Cdc7 is a client protein of Cdc37. I wondered whether or not the localization of Cdc7 was affected in *cdc37ts*. As described, Cdc7 localizes on the spindle pole body during mitosis. A proper marker was needed to point out where the spindle pole body is through the cell cycle, so that any possible change of Cdc7 localization would be detected clearly. Pcp1 is one of the spindle pole body components, which is associated with the spindle pole body throughout the cell cycle (Jin et al., 2002). The strain used was a gift from Dr Kevin Hardwick's lab, in which the *pcp1* coding sequence was fused to red-mcherry fluorescent protein (personal communication). In this strain, Pcp1 which is associated with spindle pole body though cell cycle can be traced by red fluorescence.

A Cdc7-GFP strain was sent by Dr Simanis, and the localization of Cdc7 can be observed by green fluorescence. The two strains were crossed with each other to create a strain which has the fluorescent markers in individual genes.

5.11.1 Interaction between *cdc37ts* and *cdc7-GFP*

ED1591, ED1592, ED1593 were constructed by crosses of cdc7-GFP and pcp1-red mcherry into *cdc37ts* or *cdc37*$^{+}$ backgound. Cell morphology of the three strains was checked by microscopy at permissive and restrictive temperatures. It was found that ED1592 show slightly elongation phenotype even at permissive temperature. After overnight incubation at 36 °C, ED1592 shows elongation and slight lysis phenotype (Figure 5.11). The temperature sensitive phenotype is quite different from its parent strain *cdc37-184* in which less elongation, curved cells and no lysis was observed, while the phenotype is quite similar to *cdc7-24*, indicating that Cdc7-GFP in *cdc37ts* may change its original phenotype. The GFP-tag may affect the function of Cdc7 protein in *vivo*. In the previous section, the synthetic lethal interaction between *cdc7-24* and *cdc37ts* was observed. So In *cdc7-GFP cdc37-184* (ED1592) strain, the compromised Cdc7 could interact with cdc37ts in the same way, which induces the partial Cdc7 phenotype.

Figure 5.11 Interaction between *Cdc7-GFP* and *cdc37-184*



For further investigation, nuclear and septum morphologies were observed for *cdc37-184* mutants and *cdc37*$^{+}$ cells at permissive temperature and restrictive temperature.

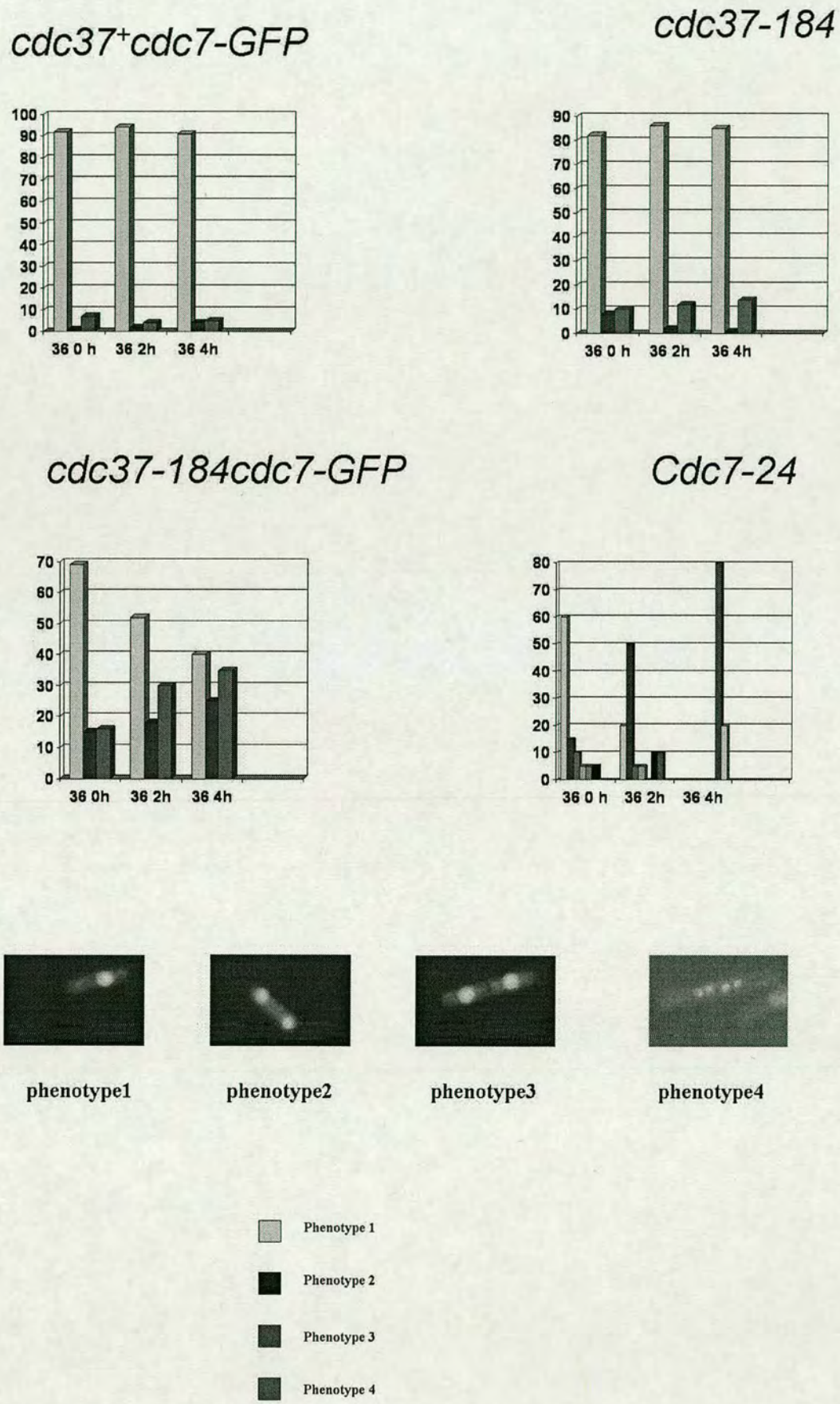
Wt, ED1592, *cdc37ts* mutant, *cdc7ts* mutant were grown overnight at 25 °C and then shifted to 36 °C. Samples were taken from cultures at 0, 2, 4 hours incubation of at 36 °C. They were fixed in 100% methanol at -20 °C and stained with Calcofluor (Chapter 2) and DAPI which can bind the yeast cell wall and nuclei separately (Streiblova et al, 1984). Cells were examined and photographed by fluoresecence microscopy. Three distinct nuclear morphology patterns could be classified. A single cell with one nucleus was defined as phenotype 1. A single cell with divided nucleus and without septum was defined as phenotype 2. Cells containing two divided nuclei and with septum were defined as phenotype 3 (Figure 5.12).

According to the observations, the *cdc37-184* mutant consists of approximately 83% phenotype 1 cells, 7% phenotype 2 cells and 10% phenotype 3 cells at permissive temperature. With time passing at restrictive temperature, the proportion of phenotype 1 slightly increased to 84%, the proportion of phenotype 2 fell (2%) and phenotype 3 cells increased to 14% (Figure5.12). It indicates that the most *cdc37* temperature-sensitive cells arrested as phenotype 1 at G2.

But the situation was quite different in ED1592 (*cdc37-184* with *cdc7-GFP*). According to my observations, it consists of 70% phenotype 1 cells, 14% phenotype 2 cells and 16% phenotype 3 cells at permissive temperature. The result is similar to *cdc7-24* at permissive temperature. On shift to 36 °C of ED1592 cells, the proportion of phenotype 1 cells decreased to 60% continuously, with a distinct increase of phenotype 2 to 18% and an increase in phenotype 3 to 30% were observed after 2 hours (Figure 5.12). After 4 hours, proportion of phenotype 1 cells decreased to 40%,

accompanied by a distinct increase of phenotype 2 cells to 25% and phenotype 3 to 35% (Figure 5.12). The increased proportion of phenotype 2 cells suggests that cells had lost the ability to form a septum, so bincleates were accumulated. It means that *Cdc7-GFP cdc37-184* cells were arrested in the stage of initiating septum formation. Cdc7 kinase activity depends on Cdc37 protein. The GFP-tag may slightly affect the function of Cdc7 protein in *vivo*. The cell with this compromised Cdc7 in a *cdc37ts* background might induce the defect of septum formation. Amazingly, the phenotype 3 cells dramatically increase, which means the cells with a defect in cleaving the septum accumulate. It is possible that the other unknown protein which controls the process of septum cleavage may also depend on Cdc37 to fulfil its function.

Figure 5.12 Statistic of 3 phenotypes in different strains



5.11.2 Localization of Cdc7 in *cdc37* mutants

Cytological research shows that Cdc7 localizes on both spindle pole bodies in early mitosis. As the spindle elongates, it can be observed at one pole of the spindle. In the *spg1-B8* mutant, no Cdc7 was detected during mitosis, suggesting that Spg1 was essential in mediating the localization of Cdc7 to the spindle pole body in early mitosis (Cerutti and Simanis, 1999). Spg1 is a GTPase of the Ras family; it was shown to be able to bind with Cdc7 *in vitro*. Although Cdc7 kinase activity is essential for initiation of septum formation, it does not depend on Spg1 function (Cerutti and Simanis, 1999).

A marker is needed to point out where the spindle pole body is through the cell cycle, so that any possible change of Cdc7 localization in *cdc37ts* mutant would be detected clearly. Pcp1 is one of the inner face spindle pole body components, which is associated with the spindle pole body throughout the cell cycle(Jin et al., 2002). For investigation of localization of Cdc7 in *cdc37ts* strains, ED1591 (*cdc7-GFP pcp1-red mcherry cdc37*$^{+}$) and ED1592 (*cdc7-GFP pcp1-red mcherry cdc37-184*), were cultured at 28 ºC and shifted to 36 ºC for 2, 3 hours. The fresh cells were fixed with pre-chilled (-20 ºC) methanol and stained with DAPI. Samples were visualized by green excitation for detecting pcp1-red mcherry signal; blue excitation for recognizing Cdc7-GFP; and UV excitation for DAPI (Figure 5.15).

At permissive temperature and restrictive temperature, four distinct morphology patterns were classified in wt cells. Pattern1: Pcp1 protein accumulates at the SPB and can be observed as a red bright dot in a cell with one nucleus, however no cdc7-GFP signal can be detected. Pattern 2: In some mononucleate cells, Pcp1 protein can be seen as two red bright dots attached to the SPB divided nucleus and no

cdc7-GFP signal. The pattern 1 cell represents cells in S phase or G2 phase or an early stage of mitosis at which the nucleus starts to divide. No cdc7-GFP signal was seen because Cdc7 only can be detected during mitosis. When two Pcp1 dots appear, it means that cells have entered metaphase and have a short spindle. It was observed that Cdc7 localizes on both spindle pole bodies in early mitosis (Sohrmann et al., 1998). In my case, no Cdc7 dots were detected in cells with two Pcp1 dots close together. A probably explanation is that the Cdc7 antibody which is used in the paper could be more sensitive to indicate Cdc7 protein accumulation in early mitosis than Cdc7-GFP.

Pattern 3: two nuclei were seen to go to the both ends of the cell by DAPI stain and without septum. Pcp1 protein attached to each of the nuclei can be observed as two red bright dots, and Cdc7-GFP signal can be detected at only one of the SPBs. The pattern 3 cell represents cells in M-G1 phase which the nuclei have divided and exist separately in one cell. One Cdc7-GFP dot can be detected in this stage for its asymmetric distribution in mitosis (Cerutti and Simanis, 1999). Pattern 4: 2 divided nuclei separated by the septum were seen. Pcp1 protein localized with each of the nuclei can be observed as two red bright dots; however Cdc7-GFP signal disappeared again. The pattern 4 cell represents cells in S phase which the nuclei are separated in different cell compartments by septum.

The same four distinct morphology patterns were also observed in *cdc37ts* strains at restrictive temperature. Cdc7-GFP signal can only be detected to localize in one of the nuclei at the stage when two Pcp1 dots were visible attached each to one nuclei. This indicates that Cdc7 can localize to SPB properly in *cdc37ts* mutants. Furthermore the proportion of the cells of the four different patterns of ED1592 cells at permissive and restrictive temperature were counted to investigate whether there were any changes at different temperature in *cdc37ts* background. The results show

Figure 5.13 Diagram of theory of Pcp1 and Cdc7 localization

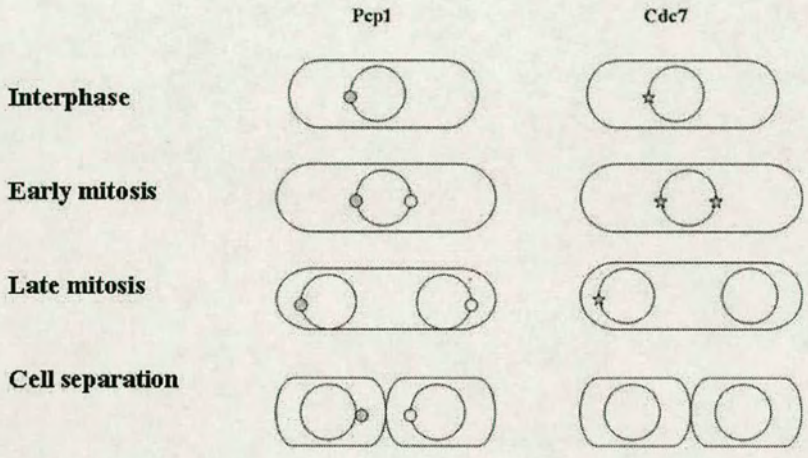
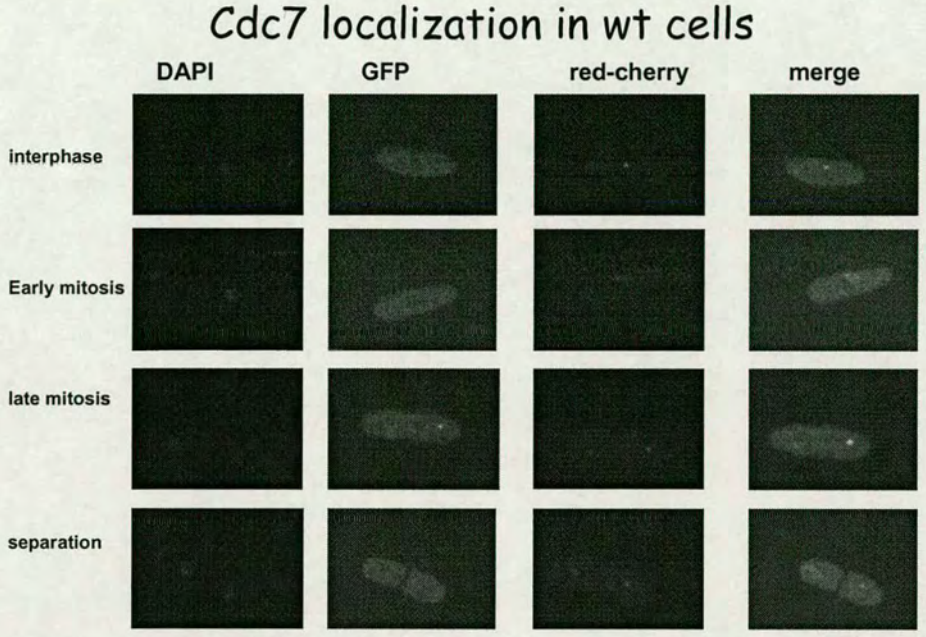
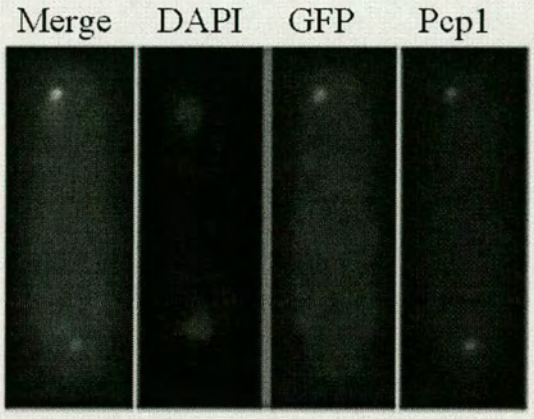


Figure 5.14 Diagram of Pcp1 and Cdc7 localization in wt cells



ED1591 cells incubated in liquid medium at 28 ℃ overnight, samples were fixed by methanol. Cdc7-GFP and Pcp1-mcherry were visualised using blue and green excitation filter sets respectively on an Intelligent Imaging Innovations. Marianas system, which incorporates a Zeiss Axiovert microscope, CoolSnap CCD, and Slidebook software.

Figure 5.15 Diagram of Pcp1 and Cdc7 localization in *cdc37ts* mutants



ED1591 cells incubated in liquid medium at 28 ℃ overnight, cells were shift to 36 ℃ for 2 hours.samples were fixed by methanol. Cdc7-GFP and Pcp1-mcherry were visualised using blue and green excitation filter sets respectively on an Intelligent Imaging Innovations. Marianas system, which incorporates a Zeiss Axiovert microscope, CoolSnap CCD, and Slidebook software.

that cell numbers of each pattern are not changed at both permissive and restrictive temperature in *cdc37ts* background (Figure 5.14; Figure 5.15) .

6.0 Conclusions and General discussion

In this project, a synthetic lethal screen was chosen to identify novel clients of Cdc37. In a synthetic lethal screen, one searches for combinations of gene mutations that together cause cell death, but that individually have little or no effect on the organism. It can be used for seeking mutations at additional loci that enhance the phenotype caused by a particular mutation.

The temperature sensitive strain *cdc37-681* was used as the starting strain. The strain was transformed with a plasmid pRep82 expressing the wild type version of *cdc37*. Two selective agents, 5-FOA and thiamine were used for screening. A total of 15 mutants were isolated out of over 40,000 colonies. 12 of 15 mutants were sensitive to both 5-FOA and thiamine. In the screening, what is of importance is to identify the mutants that can not lose the plasmid. In budding yeast, a color selective system made it easier to do this than in fission yeast. In a budding yeast synthetic lethal system, the cells that lack *ADE2* gene accumulate a red intermediate in the adenine biosynthesis pathway. Cells were transformed with a wild type copy of *ADE2* gene can be complemented the *ADE2* mutation in the chromosome, and the cells form white colonies. So plasmid loss can be found by determining the color of the colonies (Forsburg, 2001). However, in fission yeast system, it needed harder work to pick the colonies which depend on the plasmid for survival without color system.

Low frequency homologous recombination between *ura4* genes on the plasmid and chromosome may result in plasmid integration in the chromosome (Forsburg, 2001). The resulting strain would also exhibit 5-FOA sensitivity, not because of mutations which are synthetic lethal with *cdc37* and cause cell death, but because 5-FOA kills the cells which carry the integrated *ura4*$^+$ gene. To eliminate that strains

have the plasmid integrated into the chromosome, a plasmid shuffle experiment was carried out in which the *cdc37* gene was introduced on a leucine marker plasmid to see whether it could rescue the mutants. In my screen, 2 of 12 mutants could not be rescued by the lecuine plasmid; it means that plasmid had integrated.

The synthetic lethal mutations might have their own phenotype and would possibly exhibit a conditional growth defect in *cdc37* $^{+}$ background. All the candidate mutants were backcrossed with *cdc37*$^{+}$ to separate each synthetic lethal mutation out of *cdc37ts* background. Two mutants J306 and J405 did not cross. This may be caused by the additional mutations in the mutants that are required for mating. The mutations in J204 and J210 induce similar phenotypes. That is, a few cells in the colony show slight lysis and elongation at high temperature. J322 cells are quite interesting because the mutation in J322 has elongation and lysis at 36℃.

Genomic libraries made by *Hin*dIII, *Bam*HI and *Spe*I were used to transform all 10 synthetic lethal mutants. Plasmids which can rescue the mutants were isolated from 5 strains, J05, J06, J222, J218, J322. For the other strains, I failed to get the plasmid after transformations. It is possible that the genes which are able to rescue the mutants is not intact in the genomic libraries because they were cut by restriction enzymes during library construction. Or the transformations libraries were not on a large enough scale to cover genome equivalents. It may be possible to obtain more candidates genes from these mutants by transforming library or by screening more transformants.

Insert sequences from rescuing plasmids were run through a DNA BLAST search at the Sanger Centre database. *wis4*, *msc1*, *nak1* and *cdc7* were identified as candidate genes that rescue the various mutants. *wis4* encodes a MAP kinase kinase kinase (MAPKKK) and is involved in a signal transduction pathway that is activated

in under various stress conditions in *S. pombe* (Samejima et al., 1997). This stress response pathway is composed of three consecutive kinases (MAPKKK, MAPKK, and MAPK). MAPKKK phosphorylates and activates MAPKK, which in turn phosphorylates and activates MAPK. Wis4 and Win1 act as the upstream regulators of Wis1 MAPPK (Samejima et al., 1998). Wis1 is required for activation downstream of the MAPK homologue Spc1, and the Wis1–Spc1 pathway is required for survival in stressful conditions (Degols et al., 1996). Spc1 was identified as a client protein for the Cdc37 chaperone (Tatebe and Shiozaki, 2003). The mutation in *cdc37* can compromise signalling through Spc1. The protein level and the phosphorylation of Spc1 are reduced in *cdc37ts* mutant. Furthermore the physical interaction between Cdc37 protein with Spc1 was detected (Tatebe and Shiozaki, 2003).

In a related pathway in budding yeast, binding of mating pheromone to transmembrane receptors result in a series of events including the sequential activation of MAP pathway kinases Ste11, Ste7 and Fus3, leading to morphological changes. Abba-Terki et al (2000) shows that accumulation and maturation of Ste11 are defective in a *cdc37-34* background and physical interaction between Cdc37 and Ste11 were detected. It indicates that Ste11 is a direct target of Cdc37 (Abbas-Terki et al., 2000). As described above, Cdc37 is required for the activity of some components in MAP kinase cascades and some MAP kinases in yeast may likely be Cdc37 client proteins. By crossing deletion strain of *wis4* and a closely related kinase *win1* with *cdc37-681*, I showed that *wis4* and *win1* are not synthetic lethal with *cdc37-681*. This suggests that the mutated gene in chromosome is not *wis4* or *win1*. It is worth further investigating the interactions between Wis4 or Win1 and Cdc37 by biochemical means.

From J222, *msc1* was recovered as a possible candidate gene able to rescue the defect. *msc1* is a non essential gene, which was isolated as a multicopy suppressor of

a defect in *chk1*, a protein kinase in the DNA damage checkpoint pathway in fission yeast (Ahmed et al., 2004). Msc1 possesses three PHD fingers, domains commonly found in proteins that influence the structure of chromatin (Ahmed et al., 2004). Cells lacking Msc1 have a dramatically altered histone acetylation pattern. It is supposed to play an important role in regulating chromatin structure and modulating the cellular response to DNA damage (Ahmed et al., 2004). *chk1* encodes a protein kinase required for cell cycle arrest when DNA damage has occurred. It binds to and phosphorylates Cdc25, which leads to negative regulation of Cdc25 and prevents mitotic entry (O'Connell et al., 2000). It was possible that the synthetic lethal mutation is *msc1* or *chk1*. Human Cdc37 is required *in vitro* to chaperone human Chk1 in a purified chaperone system. The proposed role for Cdc37 is to recruit Hsp90 to the client by simultaneously binding the client and Hsp90 (Arlander et al., 2006). However, my results of crossing *msc1* and *chk1* deletion strains with *cdc37-681* show neither *msc1* nor *chk1* is synthetically lethal with *cdc37-681*.

The genomic fragment encoding the protein kinase *nak1* was isolated from J218. *nak1* (also known as *orb3*) encodes an essential protein kinase and plays a role in the regulation of cell polarity, growth and division. But two *nak1ts* mutants, *orb3-167* and *orb3-35/2* did not show synthetic lethality with *cdc37-681*. It would be worth trying different *cdc37 ts* mutants to check for synthetic lethal interactions.

From these results, the candidate genes can rescue the synthetic lethal mutants, but they were unlikely to be the genes corresponding to the gene mutated in the chromosome which was synthetic lethal with *cdc37-681* as I initially expected. The possible explanation will be that the candidate genes that I isolated were suppressors of the actual synthetic lethal gene in the mutant. There were several mechanisms to get suppressors during the plasmid based screening. Dosage suppressors may be isolated when they are over expressed and the gene products can stabilize the

mutated protein and help protein to fulfil its function. The most common suppressor will appear as the interaction suppressor. If two proteins can interact physically, mutating one of them might disrupt the interaction. However specific mutation of its partner may renovate the interaction and rescue the mutant. The theory of function of bypass suppressor is similar to interaction suppressor. If mutation of the gene can block the pathways which rely on the function of the gene, bypass suppressors will activate alternative pathways to replace the function of the mutated gene. Although it was hard to tell which kind of the suppressor that the candidate gene will be, the fact is that it was easy to isolate suppressor during library screening.

To test whether candidate genes were synthetic lethal with *cdc37-681*, usually deletion strains were used to cross with *cdc37ts* strains. The viability of the double mutants was observed to test the synthetic interaction of the two genes. This experiment may not reflect the actual interaction between the mutated gene and *cdc37ts* in *vivo*. I imagine that the specific mutation in the specific gene may show synthetic lethal interaction with *cdc37ts*, while the synthetic lethal interaction might not happen when the the strain with deletion of this gene was crossed with *cdc37ts*. For example, *atb2* encoding an alpha-tubulin gene, the mutation in *atb2* can give a ts phenotype, although the gene can be deleted without loss of viability (Radcliffe et al., 1998). In our case, it is possible that the mutation in *wis4, win1, msc1, chk1* of the mutant may be synthetic lethal with *cdc37ts*. The strains with deletion version of genes do not show synthetic lethal with *cdc37ts* as we had observed.

The genomic fragment containing part of *cdc7* was isolated from J322. *cdc7* encodes a protein kinase essential for cell division and plays a key role in initiation of septum formation and cytokinesis (Fankhauser C, Simanis V. 1994). Transformation of pREP42-cdc7 can rescue J322. A known cdc7*ts* mutant *cdc7-24*, shows synthetic lethality with *cdc37-681*. It suggests that defects in combination of

cdc37 and *cdc7* result in lethal phenotype. Furthermore the synthetic lethal mutation in J322 was outcrossed from *cdc37*$^{+}$ background and exhibited similar phenotype to *cdc7-24*. The synthetic lethal mutation in J322 was intercrossed with *cdc7-24,* which means that the synthetic lethal mutation of J322 lies in the *cdc7* locus Overexpression of Cdc37 protein level can overcome the *cdc7* defect, indicating that Cdc7 may need Cdc37 to fulfil its function and therefore Cdc7 may be a client of Cdc37.

The kinase activity of Cdc7 from wild type strains and Cdc2 temperature sensitive mutants remains constant through the time course after shift to 36 °C. In contrast, activity was reduced dramatically in two *cdc37ts* strains when the cultures were shifted to restrictive temperature for 1 hour, indicating that Cdc7 kinase was reduced swiftly in *cdc37ts* mutants, while Cdc7 protein levels was not affected. This indicates that Cdc7 kinase activity strongly depends on Cdc37 function.

Cdc7 localizes on both spindle pole bodies in the early mitosis, as the spindle elongates, it can be observed on one pole of the spindle (Sohrmann et al., 1998). The localization of Cdc7 in *cdc37ts* was also investigated. During construction of Cdc7-GPF in *cdc37ts* background, it was found that the temperature sensitive phenotype of *cdc7-GFP cdc37-184* is quite different from its parent strain *cdc37-184*, while its phenotype is quite similar to *cdc7-24*, indicating that Cdc7-GFP in *cdc37ts* may change its original own phenotype. In *cdc37ts* mutant, the majority of cells were arrested as phenotypel at G2 at restrictive temperature. In *cdc7-GFP cdc37-184* mutant, cells accumulated and arrested in the stage of septum formation. Amazingly, a proportion of cells were also arrested with two nuclei and a septum, which means that the cells have a defect in cleaving the septum. Cdc7 kinase activity depends on Cdc37 protein, the cell with comprised *cdc7* and *cdc37ts* would induce the defect of septum formation. The arrest of cells with two nuclei and a septum indicates that

some other unknown factors which control the process of septum cleavage may also depend on Cdc37 chaperone activity. For further investigation of localization of Cdc7 in *cdc37ts*, it was found that Cdc7-GFP signal can only be detected to localize with one Pcp1 dot when two nuclei go to the end of the cell before the septum forms. The behaviour of Cdc7 localization in *cdc37ts* was the same as in wild type cells. Moreover, the cell populations in each Cdc7 and Pcp1 localization patterns were no different between the *cdc37ts* and wild type strains. It means that Cdc7 localization was not affected in *cdc37ts* mutants.

Cdc37 has molecular chaperone activity and plays important roles in many cellular processes. A variety of biochemical techniques have been applied for identification of the possible clients of Cdc37. For example, co-immunoprecipitation (IP) is one of the most widely used techniques to identify protein interactions. In mammalian cells, Cdk4 (Dai et al 1996), Cdk6 (Lamphere et al.), Raf and v-Src (Perdew et al 1997) are examples discovered to interact with cdc37 by co-IP methods. Tandem Affinity Purification (TAP-purification) is also a powerful tool touse for identification of novel interactions. In this method, a protein of interest is fused to two affinity tags. The interacting partners as well as the fusion protein are recovered by two specific affinity purification and elution steps. MEKK1, MEKK3, TAK1 and TBK1 in human TNF-α/NF-κB signal transduction pathway were identified as Cdc37 binding proteins by TAP-purification.

Besides biochemical methods, genetic screens also act as an effective way to find genetic interactions between *cdc37* and unidentified candidates *in vivo*. Suppressor analysis is a commonly used genetic tool to identify gene interactions. CDC37 had been identified as a multi-copy suppressor of *mps1-1* in *Saccharomyces cerevisiae* by suppressor analysis (Schutz et al 1997). The fact strongly predicted that Mps1 might be a possible client of Cdc37. The interaction between Cdc37 and Mps1 was

confirmed by molecular and cytological assays (Schutz et al 1997). The cdc37-1 mutant arrests at Start with low levels of Cdc28 protein, which is predominantly unphosphorylated at Thr169, fails to bind cyclin. the levels and activity of the protein kinase Cak1 are significantly reduced in the *cdc37-1* mutant. Pulse-chase analysis indicates that Cdc28 and Cak1 proteins are both destabilized when Cdc37 function is absent (Farrell and Morgan, 2000). The similar Synthetic lethal screen to identified the interaction gene of CDC37 was carried out in budding yeast (Mort-Bontemps-Soret et al., 2002). Four protein kinases Cdc5, Cdc7, Cdc15 and Cak1 that interact with Cdc37 were identified.

cdc37 was also identified as a mutation during the genetic screen in fission yeast to identify novel components of the Spc1 SAPK cascade (Tatebe and Shiozaki, 2003). The protein level of Spc1 in the cdc37 mutant is reduced. And stress-induced phosphorylation of Spc1 by Wis1 is significantly reduced, which means that the interaction of Spc1 with Wis1 MAPKK is compromised in the cdc37 mutant. Moreover, Cdc37 physically interacts with Spc1 *in vivo*. All these results indicate that Spc1 is a client for the Cdc37 chaperone. Cdc37 may be required for stabilizing the Spc1 protein and maintaining Spc1 in a properly folded state competent for the interaction with Wis1 MAPKK (Tatebe and Shiozaki, 2003).

Cdc2 activity is reduced in *cdc37ts* mutants at restrictive temperature. Cdc2 activity in cdc37ts mutants is reduced because of its inability to maintain a stable complex with the cyclin Cdc13. Both genetic and biochemical interactions between Cdc2 and Cdc37 were detected. Mutants containing temperature-sensitive mutant alleles for both Cdc2 and Cdc37 are synthetically lethal and Cdc2 co-immunoprecipitates with Cdc37. Cdc2 may be a client of Cdc37 that relies on this molecular chaperone to promote its activation by aiding in the assembly of complexes with Cdc13 (Turnbull et al., 2006).

I identified a protein kinase Cdc7 as a candidate gene which may interact with Cdc37. Cdc7 kinase activity is reduced in *cdc37ts* mutants, but its protein level is not changed at restrictive temperature. Cdc7 localization is not affected in *cdc37ts* mutants. But compromised Cdc7 was found to interact with *cdc37ts* and result in altering *cdc37ts* phenotype. *cdc7* and *cdc37* are synthetically lethal. Cdc7 may be a client of Cdc37 that relies on this molecular chaperone to promote its function. These results emphasize the importance of Cdc37 in the activation of a large number of protein kinases.

In the future work, For the synthetic lethal strains, it is worth transforming them with different genomic libraries. This will provide more chances to recover the plasmid that may rescue the mutant. If the synthetic lethal gene is not able to be identified by plasmid complementation but it has its own obvious phenotype, gene mapping technology can be applied to determine the distance between the mutated locus and a known marker gene in the chromosome. Sequencing of the fission yeast genome is now complete, it will be easier to find where the synthetic lethal gene is through all this information. One thing that could be done is to sequence the mutant cdc7 gene in J322. It can help to know where the mutation is and what kind mutation it is and how it can affect the function of *cdc7* gene. The physical interaction between Cdc7 and Cdc37 can also considered to be done by co-immunoprecipitation. This will give me more direct evidence that Cdc37 is required for Cdc7 function.

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